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Inherited bone marrow failure syndromes in 2012

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Abstract Inherited bone marrow failure syndromes (CBMFS) are a heterogeneous group of genetic disorders characterized by bone marrow failure, congenital anomalies, and an increased risk of malignant disease. The representative diseases with trilineage involvement are Fanconi anemia and dyskeratosis congenita, while the disease with the single lineage cytopenia is Diamond–Blackfan anemia. Recent advances in our understanding of these diseases have come from the identification of genetic lesions responsible for the disease and their pathways. Although recent studies have identified many causative genes, mutations of these genes have only been found in less than half of the patients. Next-generation sequencing technologies may reveal new causative genes in these patients. Also, induced pluripotent stem cells derived from patients with CBMFS will be useful to study the pathophysiology of the diseases. The only long-term curative treatment for bone marrow failure in patients with inherited bone marrow failure syndromes is allogeneic hematopoietic stem cell transplantation, although this procedure has a risk of severe adverse effects. Multicenter prospective studies are warranted to establish appropriate conditioning regimens aimed at reducing transplant-related mortality.

Keywords Inherited bone marrow failure syndrome · Fanconi anemia · Diamond–Blackfan anemia · Dyskeratosis congenita

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Introduction

Inherited bone marrow failure syndromes (IBMFS) are a heterogeneous group of genetic disorders characterized by bone marrow failure, congenital anomalies, and increased risk of malignant disease. Such bone marrow failure may affect all three hematopoietic cell lineages or single cell lineages individually. Diseases characterized by trilineage involvement include Fanconi anemia and dyskeratosis congenita, while Diamond–Blackfan anemia results in single-lineage cytopenia. Recent advances in our understanding of these diseases have arisen from the identification of genetic lesions responsible for such diseases and their pathogenic pathways. These investigations have further clarified both normal and pathological hematopoiesis. In this current review, we describe recent insights into three IBMFS: Fanconi anemia, Diamond–Blackfan anemia, and dyskeratosis congenita.

Fanconi anemia

Fanconi anemia (FA) is a rare autosomal recessive disease characterized by congenital abnormalities, progressive bone marrow failure, and cancer susceptibility. FA, which has an incidence of less than 10 per million live births, is the most frequent inherited cause of aplastic anemia [1]. FA is a genetically heterogeneous disease defined by complementation groups. To date, 15 genes have been identified as playing a causative in FA and these genes, FANCA to FANCP, have been cloned [2]. Children with FA often develop aplastic anemia during the first decade of life, with death often resulting from complications of bone marrow failure, such as severe infection or bleeding. FA

patients also develop clonal chromosomal abnormalities in bone-marrow progenitor cells, such as monosomy 7, which are associated with myelodysplastic syndrome (MDS) and acute myeloblastic leukemia (AML) [3]. The gene FANCD1, which is responsible for complementation group FA-D1, is identical to the hereditary breast cancer susceptibility gene, BRCA2, and has been reported as affected in 3 % of patients with Fanconi anemia. As compared with children from other FA groups, more severe phenotypes are seen in FA-D1 patients, such as co-occurrence of multiple anomalies, development of multiple malignancies with earlier onset, and increased incidence of leukemia and solid tumors [4], including Wilms tumor, neuroblastoma, and brain tumors.

While the treatment of choice for FA patients remains allogeneic stem cell transplantation (SCT) from an HLA-matched sibling or unrelated donor, older patients may develop squamous-cell carcinomas (SCCs) of the head and neck or gynecological system. In particular, some studies have demonstrated there is a high incidence of SCC, such as esophageal cancer, in FA patients who have received SCT. The age-specific hazard of SCC has been shown to be 4.4-fold higher in patients who receive transplants and, in addition, SCCs occurred at significantly younger ages in the transplant group [5]. Thus, further investigations of the complete care of FA patients need to be undertaken.

Complementation groups and genes of FA

Since cells derived from FA patients are hypersensitive to DNA interstrand cross-linking (ICL) agents, such as diepoxybutane (DEB), mitomycin C (MMC), and cisplatin, it is expected that FA genes are involved in ICL repair. In 1993, FANCC was the first FA gene to be cloned by expression cloning [6, 7]. Subsequently, 15 other genes have been cloned (Table 1). At present, the FANC genes, which range from FANC"A" to FANC"P", and the FA pathway have been shown to resolve ICLs encountered during DNA replication. There are three primary groups of FA proteins, which include the FA core complex, the ID (FANCD2/I) complex, and the BRCA complex (Fig. 1). In these groups, there are eight FA proteins (FANCA/B/C/E/F/G/L/M) that form a multi-subunit ubiquitin E3 ligase complex, the FA core complex, which activates the monoubiquitination of the ID complex after genotoxic stress, such as ICL, or during the S phase (Fig. 1) [8, 9]. The monoubiquitinated ID complex forms foci on damaged DNA. FANCM is also a crucial gene, as it is a sensor for detecting stalled DNA replication. The BRCA complex, which is also referred to as homologous recombination (HR), consists of FANCD1, FANCJ, FANCN, and FANCO, and is located downstream of the DI complex on the ICL repair pathway.

Table 1 Genes mutated in patients with Fanconi anemia

	Other names	Chromosomal Locus	Population in FA patients (%)
FANCA		16q24.3	60–70
FANCB		Xp22.2	2
FANCC		9q22.3	14
FANCD1	BRCA2	13q13.1	3
FANCD2		3p25.3	3
FANCE		6p22-p21	3
FANCF		11p15	2
FANCG		9p13	10
FANCI		15q25-q26	1
FANCJ	BACH1/BRIP1	17q22	2
FANCL		2p16.1	0.20
FANCM		14q21.3	0.20
FANCN	PALB2	16p12.3-p12.2	0.70
FANCO	RAD51C	17q22	0.20
FANCP	SLX4	16p13.3	0.20

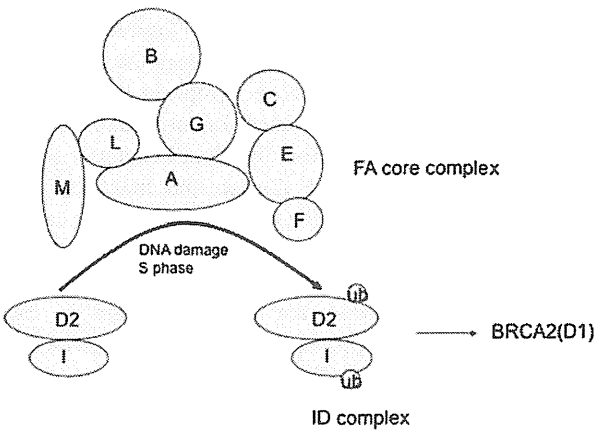

Fig. 1 Simplified scheme of the FA pathway. Depending on the FA core complex, FANCD2 and FANCI are monoubiquitinated after DNA damage or during the S-phase

Prognosis factors in FA patients

Although FANC gene knockout mice models have been established, they differ from the hematological phenotypes of human FA patients [10]. With the exception of the lethal phenotype of the BRCA2/FANCD1 knockout mouse, the hematological parameters of the other FA groups show only a slightly decreased platelet count and a slightly increased erythrocyte mean cell volume in mice at a young age, which did not progress to aplastic anemia or leukemia. However, both male and female mice showed hypogonadism and impaired fertility, which is consistent with the human FA patient phenotypes.

Recent studies have revealed a relationship between the acetaldehyde and FA pathways. Acetaldehyde is an organic chemical compound that is naturally present in coffee, bread, and ripe fruit, which is produced as product of a plant's normal metabolism. It is also produced by the oxidation of ethylene. In the liver, the enzyme alcohol dehydrogenase (ADH) oxidizes ethanol into acetaldehyde, which is then further oxidized into harmless acetic acid by acetaldehyde dehydrogenase (ALDH). These oxidation reactions are coupled with the reduction of NAD^+ to NADH. ALDH2, an isozyme of ALDH, contains the functional polymorphism, *ALDH2* Glu487Lys. An association between this polymorphism and squamous cell carcinomas, such as esophageal cancer in alcoholics, has been reported. A recent study reported that exposure of cells to acetaldehyde results in a concentration-dependent increase in FANCD2 monoubiquitination [11]. Acetaldehyde also stimulates BRCA1 phosphorylation at Ser1524 and increases the level of H2AX, a marker of homologous recombination. Both modifications occur in a dose-dependent manner.

Another report showed that ALDH2 is essential for the development of FANCD 2(−/−) embryos [12]. Nevertheless, mothers with AA enzyme (ALDH 2(+/−)) can support the development of double-mutant (ALDH2 (−/−)FANCD2(−/−)) mice. These embryos are unusually sensitive to ethanol exposure in utero, with ethanol consumption by postnatal double-deficient mice rapidly precipitating bone marrow failure. ALDH2 (−/−)FANCD2(−/−) mice also spontaneously develop acute leukemia.

This previous study also provided the first evidence of the factors responsible for driving the FA hematological phenotype in mice. DNA damage caused by acetaldehyde may contribute critically to the genesis of fetal alcohol syndrome in fetuses, as well as to abnormal development, bone marrow failure, and cancer predisposition in FA patients. This research group also focused on hematopoietic stem cells (HSCs) in another study [13]. They reported finding that some aged ALDH2(−/−)FANCD2(−/−) mutant mice that did not develop leukemia spontaneously developed aplastic anemia, with a concomitant accumulation of damaged DNA within the hematopoietic stem and progenitor cell (HSPC) pool. Only HSPCs and not the more mature blood precursors require Aldh2 for protection against acetaldehyde toxicity. There is more than a 600-fold reduction in the HSC pool of mice deficient in both FA pathway-mediated DNA repair and acetaldehyde detoxification. This study data indicated that the emergence of bone marrow failure in FA was probably due to aldehyde-mediated genotoxicity restricted to the HSPC pool.

All of the ALDH data suggest that ALDH2 polymorphism is critical to the prognosis of FA patients.

Intercrosslink repair

DNA ICLs are toxic to dividing cells, as they induce mutations, chromosomal rearrangements, and cell death. In order to survive, organisms have developed strategies for dealing with DNA damage. As such, specialized repair pathways have evolved for specific kinds of DNA damage, including double-strand break (DSB) and ICL. Inducers of ICLs are important drugs in cancer treatment and include the well-known chemotherapeutic agents mitomycin C, cisplatin, cyclophosphamide, and their respective derivatives. While cells derived from most individuals with FA are hypersensitive to ICLs, they are generally not hypersensitive to inducers of DSBs such as ionizing radiation, indicating that the ICL repair pathway is distinct from that of DSB.

Homologous recombination is a DNA repair pathway that utilizes strand exchange in a gene conversion reaction involving a single-strand and a DNA duplex. In mammalian cells, this is a major repair pathway for DNA damage such as DSBs. The strand exchange protein RAD51 and the products of the hereditary breast cancer susceptibility genes BRCA1 and BRCA2 [14, 15] are critical proteins in HR in mammalian cells.

In 2005, a cellular study in humans showed that mutation of either the FA core complex members or the FANCD2 monoubiquitination site resulted in HR defects [16]. These defects, however, are mild compared with those resulting from a BRCA2 deficiency. HR measurements in these previous studies were performed with the widely used DR-GFP reporter system, in which a DSB formed by I-SceI endonuclease results in green fluorescent protein-expressing (GFP+) cells repaired by HR (Fig. 2). Further studies have reported on the mechanisms of ICL repair, particularly in terms of the replication-coupled manner. A 2008 study using a cell-free system based on *Xenopus* egg extracts found that ICL is repaired in a replication-dependent manner [17]. Another study in the *Xenopus* egg showed that ubiquitinated FANCI-FANCD2 is essential for replication-dependent ICL repair and that it is able to control the incision step [18]. Development and use of a TR-GFP assay, a modified version of the DR-GFP HR assay system, demonstrated that ICL repair in mammalian cells is dependent on DNA replication. The TR-GFP assay uses a DNA template with a site-specific ICL at sequences that are complemented to triplex-forming oligonucleotide conjugated with psoralen (pso-TFO) [19]. The construct also contains an origin of replication from the Epstein–Barr virus (EBV), enabling replication in human cells. Their results showed that ICL-induced HR was substantially compromised in the absence of FA proteins, suggesting that the FA pathway is specifically involved in replication-coupled HR repair. Use of direct assays for ICL-induced HR in vivo, along with studies that

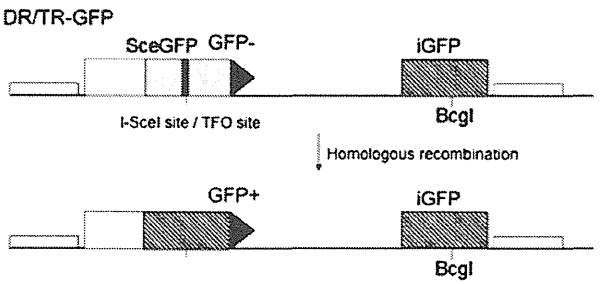

Fig. 2 DR-GFP assay. In the *DR-GFP* substrate, an I-SceI site is inserted into the GFP gene on sceGFP. GFP is inactivated by the stop-codon in the I-SceI site. To restore functionality of the *GFP* gene, the *iGFP* gene has .8 kb of sequence homology to direct the repair of an I-*Sce*I-cleaved *SceGFP* gene

have demonstrated the involvement of the FA pathway overall, may facilitate delineation of the mechanisms and factors involved in this process.

Diamond–Blackfan anemia

Diamond–Blackfan anemia (DBA) is a rare congenital bone marrow failure syndrome characterized by severe normochromic macrocytic anemia and reticulocytopenia, with selective hypoplasia of erythroid precursors in the bone marrow. Up to 50 % of affected individuals have physical abnormalities including short stature, craniofacial dysmorphism, heart defects, and anomalies of the thumbs and genitourinary tract [20]. Increased risk of malignant disease, such as acute myeloid leukemia and osteogenic sarcoma, has also been reported to occur in this syndrome [21]. The incidence of DBA has been estimated to be 5–7 per million live births in Europe and North America. In a national study conducted in Japan between 2006 and 2010, 65 new DBA patients were registered. During this study period, the mean number of live births per year in Japan was reported to be 1.08 million, putting the incidence of DBA at 12 per each million live births, as most of these patients were diagnosed as DBA during infancy. The majority of these patients are sporadic, with the percentages of patients with autosomal dominant inheritance reported to be less than 10 %. Corticosteroids are recommended as a first line therapy, as these have been reported to improve erythropoiesis in approximately 80 % of DBA patients. In patients refractory to corticosteroids or who develop other forms of cytopenia, HSC transplantation has been suggested as a viable alternative [22].

Molecular pathogenesis

The first DBA gene (RPS19) was identified in 1999 and was found in approximately 25 % of the probands in western countries [23]. Since then, a total of nine genes encoding large (RPL) or small (RPS) ribosomal subunit proteins were found to be mutated in DBA patients, including RPL5 (6.6 %), RPL11 (4.8 %), RPL35A (3 %), RPS24 (2 %), RPS17 (1 %), RPS7 (1 %), RPS10 (6.4 %), and RPS26 (2.6 %) [24] (Table 2). Collectively, mutations in at least one of these nine genes have been detected in approximately 50–60 % of DBA patients. Of 68 Japanese been examined, mutations in RPS 19, RPL5, RPL11, RPS17, RPS26 were identified in 10 (14.7 %), six (8.8 %), three (4.4 %), one (1.5 %), one (1.5 %), and one (1.5 %), respectively. These mutations have subsequently been determined to occur in 32.4 % of Japanese patients [25, 26]. A low incidence of mutations in the RPS19 gene may account for the overall lower incidence of total mutations in the Japanese population.

As conventional gene sequencing cannot identify large gene deletions, there have been only a few reports of patients with allelic losses in the RPS19 and RPL35A genes. Kuramitsu et al. [26] investigated large deletions of the RP genes using gene copy number variation analysis based on a quantitative-PCR and a single-nucleotide polymorphism (SNP) array. This study used sequencing to screen for large gene deletion in 27 patients without gene mutations. The PCR-based gene copy number assay identified a large deletion in seven (25.9 %) of 27 patients. Of these, three patients had RPS17, two had RPS19, one had RPL5, while one had RPL35A deletions. The SNP array confirmed six of the seven large deletions. Based on these new methods, the frequency of RP gene abnormalities in the DBA patients increased to 42.6 %. All patients with large deletions in DBA genes exhibited malformation with growth retardation. However, half of the patients with a mutation due to sequencing had growth retardation, while all seven patients with a large deletion exhibited growth retardation. While four of seven patients responded to corticosteroids, there were no phenotypic

Table 2 Ribosomal protein gene mutations and deletions in 68 Japanese patients with Diamond–Blackfan anemia

Gene	Mutation	Deletion	Total (%)
RPS 19	10	2	12 (17.6 %)
RPL 5	6	1	7 (10.3 %)
RPS 17	3	0	3 (4.4 %)
RPL 11	3	1	4 (5.9 %)
RPS 10	1	0	1 (1.5 %)
RPS 26	1	0	1 (1.5 %)
RPS 35A	0	1	1 (1.5 %)
RPS 24	0	0	0
RPS 14	0	0	0
Total	22	7	29 (42.6 %)

differences noted between patients with and without large deletions, including response rate to corticosteroids and other malformations.

Farrar et al. [27] also identified RP gene deletions in nine (17 %) of 51 patients without any identifiable mutation by SNP array. Of these nine patients, three had RPS17, two had RPS26, two had RPS19, and two had RPL35A deletions. Clinically, five of the nine patients responded to corticosteroids. Two exhibited short stature. These two studies suggested that genomic deletions may be detected in 4–10 % of DBA patients, which is more common than has been previously suspected. Thus, in addition to conventional gene sequencing, molecular studies of suspected DBA cases should also include either a SNP array or PCR-based gene copy number assay.

Despite extensive sequencing of all the RP genes, at present mutations have only been found in approximately half of DBA patients examined, which raises the question whether other genes are responsible for DBA. Recent advance in genomic sequencing have made it possible to search for new candidate genes. Sankaran et al. [28] performed exome sequencing on two siblings without RP gene mutations. Both affected siblings satisfied the diagnostic criteria for DBA and both parents had normal blood values, suggesting X-linked or autosomal recessive inheritance. During sequencing, at least 10-fold coverage was obtained in more than 93 % of the target bases. After filtering, a total of 74 variants were identified as being shared by the three affected siblings. Of these 74 mutations, 31 were found in two affected siblings but not in the unaffected sibling. No variants were identified that would fit an autosomal recessive model of inheritance. Only the GATA1 gene showed appropriate segregation for an X-linked disease with full penetrance. The mutation in the GATA1 gene is a G-C transversion at position 48,649,736 on the X chromosome and results in a substitution of leucine for valine at amino acid 74 of the GATA1 protein. This mutation impaired production of the full-length form of the exon 2 protein. After screening 62 additional male DBA patients without known mutations for the GATA1 mutation, the study also identified one patient with a mutation in GATA1 at the exon 2-intron 2 junction. It was predicted that this would result in impaired splicing and a frameshift of the full-length GATA1 open reading frame. Overall, this study has opened new avenues for studying the molecular pathogenesis of DBA.

Role of p53 in the pathophysiology of DBA

Although current evidence suggests that impaired ribosomal biogenesis should affect all blood cell lineages, one question remains as to why it affects only the erythroid progenitors. Several animal models have demonstrated the role of p53 in the pathophysiology of DBA. The RPS19-deficient zebrafish model has been shown to have many features of DBA and is accompanied by the up-regulation of the p53 family [29]. Suppression of p53 in the RPS19-deficient zebrafish alleviated the phenotype and improved survival. RPS19 knockdown mouse fetal liver cells, which were created by retrovirus-infected siRNA, showed reduced proliferation but normal differentiation of erythroid cells and an increased level of p53 and p21 [30]. Dutt et al. [31] have examined the accumulation and activity of p53 in different hematopoietic lineages after a partial knockdown of the RPS19 gene in primary human bone marrow-derived CD34 cells. Their study showed that p53 accumulates selectively in erythroid progenitors, resulting in lineage-specific p53 target gene expression, cell cycle arrest, and apoptosis. While pifithrin-α has been shown to inhibit the activity of p53, nutlin-3 activates p53 through the inhibition of HDM2. In addition, nutlin-3 selectivity impairs erythropoiesis, whereas inhibition of p53 by pifithrin-α rescues the erythroid defect. To directly examine whether p53 accumulation is operative in patients with DBA, bone marrow biopsies from eight patients with DBA were stained with anti-human p53 antibody and shown to have strong nuclear staining in two patients and weak nuclear staining in six patients. The erythroid lineage has a low threshold for the induction of p53, which accounts for the selective impaired erythropoiesis in patients with DBA.

Alternative therapies for DBA

Although approximately 80 % of DBA patients initially respond to corticosteroid, half of the responders are steroid-dependent. Only 20 % of these patients achieve remission. Although historically many alternative drugs have been tried, there has been no agreement on a second-line therapy. L-leucine is an essential amino acid and is known to be an activator of mRNA and stimulate protein synthesis through the mammalian target of rapamycin (mTOR) pathway. L-leucine treatment of the RPS19-deficient zebrafish model results in a striking improvement of anemia and developmental defects. These findings were reproduced in primary human CD34 cells after knockdown of the RPS19 gene [32]. Therapeutic effect of L-leucine has also been confirmed in the mouse model for RPS19-deficient DBA and shown to be associated with reduced p53 activity in hematopoietic progenitors [33]. Recently, leucine has been used on an investigational basis in one patient with DBA and is reported to have achieved a remission [34].

These findings support commencement of a clinical trial with L-leucine as an alternative therapy for DBA.

Dyskeratosis congenita

Clinical features of patients with dyskeratosis congenita

Dyskeratosis congenita (DC) is a rare inherited disease characterized by the classical mucocutaneous triad of abnormal skin pigmentation, nail dystrophy, and mucosal leucoplakia in approximately 80–90 % of patients [34]. Patients with DC are unable to maintain the telomere complex that protects the chromosome ends and consequently have very short telomeres [35]. Shortened telomeres can cause a wide variety of clinical features across a phenotypic spectrum consisting not only of mucocutaneous abnormalities but also multisystem symptoms including bone marrow failure, pulmonary fibrosis, hepatic fibrosis, and predisposition to malignancy [36, 37]. Indeed, non-mucocutaneous features, such as bone marrow failure and pulmonary fibrosis, occasionally precede mucocutaneous abnormalities, making it difficult to diagnose patients with DC based on clinical features alone. The incidence of DC is estimated to be one per million live births.

The diagnostic criteria for DC proposed by Vulliamy [38] include one or more of the three classic mucocutaneous features combined with hypoplastic bone marrow and at least two other somatic features known to occur in DC. The primary causes of mortality in patients with DC are bone marrow failure syndrome (60–70 %), pulmonary complications (10–15 %), and malignancy (particularly MDS and AML) (10 %) [36, 37].

Genetic background of DC

DC is a genetically heterogeneous disorder, showing autosomal recessive, autosomal dominant, and X-linked inheritance. The *DKC1* gene on chromosome (chr) Xq28, which encodes dyskerin, was the first gene identified in the X-linked DC patients [39]. Dyskerin has a close association with the RNA component of telomerase (*TERC*), and mutations in dyskerin cause a reduction in accumulation of *TERC* and reduced telomere length [35]. In addition to its role in the biogenesis of telomerase RNA dyskerin is involved in ribosomal RNA biogenesis. Dyskerin catalyzes uridine to pseudouridine, which is a critical step for ribosomal RNA maturation and function. These findings imply that both telomere and ribosomal defects may occur in patients with *DKC1* mutations. Subsequently, heterozygous *TERC* mutations have also been found in autosomal dominant DC patients [40]. Genetic screening has identified mutations of other components of the telomerase complex, including *TERT* (chr 5p15) [41, 42], *NOP10* (chr 15q14-q15) [43], and *NHP2* (chr 5q35) [44] in patients with rare autosomal recessive DC. Mutations of *TERT* have also been reported in the autosomal dominant family [45]. Moreover, heterozygous mutations of *TINF2* (chr 14q12) that encode TIN2, which is the main component of shelterin and which protects telomeres, have been identified in <11 % of DC patients [46, 47].

More recently, mutations of *TCAB1* (chr 17p13) were identified in patients with DC as autosomal recessive forms [48]. Venteicher et al. [49] found that TCAB1 associates with TERT, dyskerin and TERC, and small Cajal body RNAs (scaRNAs) that are involved in modifying splicing RNAs to control telomerase trafficking. TCAB1 defects prevented TERC from associating with the Cajal bodies, which disrupted the telomerase–telomere association. A recent case report described biallelic mutations of the *CTC1* gene (chr 17p13) in a patient with DC [50]. This gene was originally described as causative gene of the Coats plus syndrome, which is a form of cerebroretinal microangiopathy with calcifications and cysts (CRMCC). The mutation frequencies of these new genes for DC remain unknown.

At present, eight of the mutated genes in DC have been shown to be associated with the telomerase holoenzyme (TERT, *TERC, DKC1, NOP10, NHP2, TCAB1,* and *CTC1)* or the shelterin complex (TINF2), accounting for approximately 50 % of DC patients. Mutations in telomerase and telomere components have also been identified in patients with aplastic anemia, pulmonary fibrosis, and liver diseases that did not have any mucocutaneous manifestations [45, 46, 51–59]. These findings suggest that defective telomere maintenance causes not only classical DC, but also a broad spectrum of diseases previously thought to be idiopathic and thus this has led to a new concept of diseases termed ''syndromes of telomere shortening''.

Cryptic DC patients in aplastic anemia

Patients with DC have been shown to have disease diversity in terms of age at onset, symptoms, and severity. This diversity occurs even among the patients with the same gene mutation. Bone marrow failure sometimes precedes mucocutaneous manifestations in patients with DC, and a substantial proportion of patients with aplastic anemia have shorter telomeres compared with normal individuals [60, 61]. These observations have prompted screening for gene mutations responsible for telomere maintenance in patients with aplastic anemia and other bone marrow failure syndromes. This screening identified mutations in *TERC* and *TERT* in 3 % of the aplastic anemia patients [54, 55]. Our research group conducted a study in Japanese children with aplastic anemia and identified two of 96 as having the *TERT* mutations, although none of the patients had a *TERC* mutation [53]. Patients with *TERC* or *TERT* mutations have been shown to have very short telomeres in their blood cells. Recently, Du et al. [52] found that 6 (5.5 %) of 109

pediatric patients with severe aplastic anemia had mutations of *TINF2*. In an unpublished study, our research group screened for mutations of *TINF2* and found that of the 96 pediatric patients with aplastic anemia that were examined, none exhibited any mutations of this gene.

Three methods are commonly used for measuring telomere length, including Southern blot, real-time polymerase chain reaction, and flow cytometry and fluorescence in situ hybridization (flow-FISH). Of these, the flow-FISH has been shown to be the most appropriate when undertaking "prospective" screening [62, 63]. As shown in Fig. 3, patients with DC and aplastic anemia with the *TERT* mutation were all found to have very short telomeres as compared with the idiopathic aplastic anemia patients and normal individuals. As a small subset of patients with apparently idiopathic aplastic anemia have been shown to carry telomere gene mutations, identification of such patients is critical for informing treatment decisions. Aplastic anemia patients should be routinely screened for telomere gene mutations prior to starting any treatment. However, because screening of gene mutations can be both laborious and time consuming, we have adopted the screening of telomere length in blood cells rather than screening of gene mutations.

It should be noted that short telomeres are not specific for patients with DC, as they are also seen in patients with other bone marrow failure syndromes. Although short telomeres have also been found in patients with other congenital bone marrow failure syndromes, such as Shwachman–Diamond syndrome and Fanconi anemia, telomere lengths in patients with DC have been demonstrated to be shorter than those in all other bone marrow failure syndromes. In fact, telomere length in most patients with DC is below the first percentile of telomere length found in healthy controls [64].

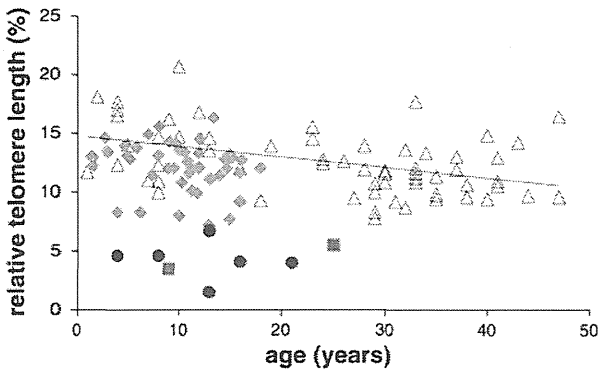

Fig. 3 Relative telomere length in peripheral blood lymphocytes from patients with dyskeratosis congenita (*filled circles*), patients with aplastic anemia harboring TERT mutations (*filled squares*), patients with idiopathic aplastic anemia (*filled argyles*) and normal individuals (*open triangles*). Telomere lengths were measured by flow cytometry-fluorescent in situ hybridization (flow-FISH). Relative telomere length was calculated as the ratio between the telomere signal of each sample and the telomere signal of the control cell line (cell line 1301). These data were provided by the Department of Pediatrics, Nagoya University Graduate School of Medicine

Family members of patients with DC should receive genetic counseling to rule out if they are silent carriers. In particular, genetic counseling is necessary during the proband search for a donor for HSC transplantation. Studies on telomere length analyses in families with DC have shown that mutated carriers with clinical signs of bone marrow failure have short telomeres. Even so, telomere length cannot predict the presence or absence of a mutation in family members with bone marrow failure. In addition, there have been rare cases that show normal telomere length, even though the subject harbors the same mutation as the proband. This suggests that mutation alone does not sufficiently explain the reduction of telomere length [51].

Clinical management for DC

Bone marrow failure and immune deficiency are the most common causes of death in up to 60–70 % of patients with DC. Androgen (e.g. oxymetholone) has been used to improve cytopenia in patients with DC since the 1960s. However, the mechanism of action of androgen has not been well understood until recently. Calado et al. [65] showed that in vitro exposure of normal peripheral blood cells to androgen produced higher TERT mRNA levels. When these patients were treated with cells from patients who had a heterozygous mutation of the telomerase, it was possible to restore their low baseline telomerase activity to normal levels. Thus, as telomere shortening is closely associated with malignant disease, androgen therapy might be able to prevent or postpone the development of various types of cancers. Erythropoietin and/or G-CSF combined with androgen has occasionally provided transient hematopoietic recovery to poor responders to androgen alone [66]. However, this combination should be used with caution, as severe splenic peliosis and fatal rupture have been reported in two patients with DC who received simultaneous administration of androgen and G-CSF [67].

Allogeneic HSC transplantation is the only curative treatment for bone marrow failure in patients with DC. However, the outcome in previous reports has been disappointing due to unacceptable transplant-related toxicities, including severe pulmonary/liver complications, especially in transplants from an alternative donor [68, 69]. To avoid these complications, non-myeloablative conditioning regimens have been recently used in several cases. Dietz et al. [70] reported encouraging results of six patients with DC who received a fludarabine-based non-myeloablative regimen. Of the four surviving patients, three were recipients of unrelated grafts. Non-myeloablative

transplants are expected to provide improvement in the short-term survival. At our institute, three patients with DC underwent allogeneic bone marrow transplantation following non-myeloablative conditioning from 2003 to 2009. Successful engraftment was achieved in all patients with only a few regimen-related toxicities, and at the present time all continue to survive without any symptoms [71]. However, due the late effects of conditioning agents and allogeneic immune responses within the recipient's organs, such as the lung and liver, longer-term follow-ups are necessary to definitively clarify the present results.

Conclusion

Although recent studies have identified many causative genes, mutations of these genes have only been found in half of the patients with DBA or DKC. Next-generation sequencing (or massive parallel sequencing) technologies have led to a tremendous revolution in genomics, with their effects currently becoming increasingly widespread. This new strategy may soon be able to reveal the remaining unknown causative genes in IBMFS.

Recently, Agarwal et al. [72] established induced pluripotent stem cells (iPSCs) derived from a patient with DC and showed that the reprogrammed DC cells overcame a critical limitation in TERC levels to restore the telomere maintenance and self-renewal. These findings indicate that drugs or gene therapy that upregulate TERC activity may show therapeutic potential in patients with DC. These same strategies may also be applicable for other IBMFS.

The only long-term curative treatment for bone marrow failure in patients with IBMFS is allogeneic HSC transplantation, although this procedure has a risk of severe adverse effects. Multicenter prospective studies are needed to establish appropriate conditioning regimens aimed at reducing transplant-related mortality. Future studies must aim to improve short-term outcomes, such as hematological recovery, and to decrease the incidence of late adverse effects.

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To the editor:

Rabbit antithymocyte globulin and cyclosporine as first-line therapy for children with acquired aplastic anemia

Horse antithymocyte globulin (hATG) and cyclosporine have been used as standard therapy for children with acquired aplastic anemia (AA) for whom an HLA-matched family donor is unavailable. However, in 2009, hATG (lymphoglobulin; Genzyme) was withdrawn and replaced by rabbit ATG (rATG; thymoglobulin; Genzyme) in Japan. Many other countries in Europe and Asia are facing the same situation.[1] Marsh et al recently reported outcomes for 35 adult patients with AA who were treated with rATG and cyclosporine as a first-line therapy.[2] Although the hematologic response rate was 40% at 6 months, several patients subsequently achieved late responses. The best response rate was 60% compared with 67% in a matched-pair control group of 105 patients treated with hATG. The overall and transplantation-free survival rates appeared to be significantly inferior with rATG compared with hATG at 68% versus 86% ($P = .009$) and 52% versus 76% ($P = .002$), respectively. These results are comparable to those from a prospective randomized study reported by Scheinberg et al comparing hATG and rATG.[3] Both studies showed the superiority of hATG over rATG.[2,3]

We recently analyzed outcomes for 40 Japanese children (median age, 9 years; range, 1-15) with AA treated using rATG and cyclosporine. The median interval from diagnosis to treatment was 22 days (range, 1-203). The numbers of patients with very severe, severe, and nonsevere disease were 14, 10, and 16, respectively. The ATG dose was 3.5 mg/kg/day for 5 days. The median follow-up time for all patients was 22 months (range, 6-38). At 3 months, no patients had achieved a complete response (CR) and partial response (PR) was seen in only 8 patients (20.0%). At 6 months, the numbers of patients with CR and PR were 2 (5.0%) and 17 (42.5%), respectively. After 6 months, 5 patients with PR at 6 months had achieved CR and 4 patients with no response at 6 months had achieved PR, offering a total best response rate of 57.5%. Two patients relapsed at 16 and 19 months without receiving any second-line treatments. Two patients with no response received a second course of rATG at 13 and 17 months, but neither responded. Sixteen patients underwent hematopoietic stem cell transplantation (HSCT) from alternative donors (HLA-matched unrelated donors, n = 13; HLA-mismatched family donors, n = 3). Two deaths occurred after rATG therapy, but no patients died after HSCT. Causes of death were intracranial hemorrhage at 6 months and acute respiratory distress syndrome at 17 months. The overall 2-year survival rate was 93.8% and the 2-year transplantation-free survival rate was 50.3% (Figure 1).

In our previous prospective studies with hATG, the response rates after 6 months were 68% and 70%, respectively, with no increases in response rates observed after 6 months.[4,5] Our results support the notion that rATG is inferior to hATG for the treatment of AA in children. First-line HSCT from an alternative donor may be justified, considering the excellent outcomes in children who received salvage therapies using alternative donor HSCT.

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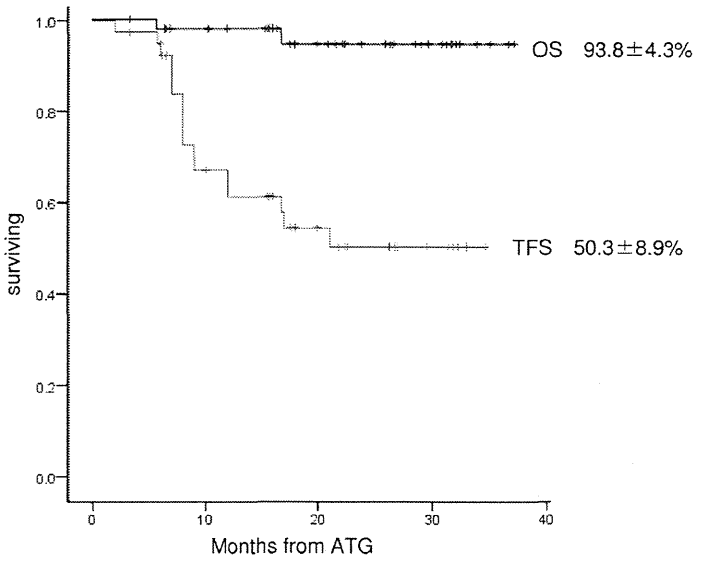

Figure 1. Kaplan-Meier estimates of overall survival (OS) and transplantation-free survival (TFS) in 40 Japanese children with AA. Survival was investigated using Kaplan-Meier methods. OS for all patients with AA after rATG and cyclosporine as first-line therapy included patients who later received HSCT for nonresponse to rATG. In the analysis of TFS for all patients treated with rATG and CSA, transplantation was considered an event.

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To the editor:

Peripheral blood stem cells versus bone marrow in pediatric unrelated donor stem cell transplantation

The relative benefits and risks of peripheral blood stem cells (PBSCs) versus bone marrow (BM) for allogeneic hematopoietic stem cell transplantation (SCT) are still a matter of highly controversial debates.[1-3] The first randomized study comparing the 2 stem cell sources in unrelated donor SCT recently documented comparable overall and event-free survival, but indicated a higher risk for chronic graft-versus-host disease (GVHD) with PBSCs.[4] Only a few pediatric patients were included in this study even though the long-term sequelae of chronic GVHD are of particular concern in this patient group.

We retrospectively compared the long-term outcome of contemporaneous unrelated donor SCT in 220 children transplanted with BM (n = 102) or PBSCs (n = 118) for hematologic malignancies and reported to the German/Austrian pediatric registry for SCT. All patients had received myeloablative conditioning followed by unmanipulated SCT from HLA-matched unrelated donors. The PBSC and BM groups were comparable with regard to patient and donor age, sex, cytomegalovirus (CMV) serostatus, disease status at transplantation, GVHD prophylaxis, growth factor use, and degree of HLA matching. The groups differed with regard to disease category with slightly more myelodysplastic syndrome patients ($P = .02$) and a higher CD34-cell dose ($P = .001$) in the PBSC group.

Neutrophil and platelet engraftment were achieved significantly faster after PBSC than BM transplantation (Figure 1A-B). In this entirely pediatric cohort, the incidence of clinically relevant grade II-IV acute GVHD (Figure 1C) did not differ. Most importantly, the incidence of chronic GVHD (PBSCs vs BM: 35% vs 33%, respectively; $P = .9$) and extensive chronic GVHD (Figure 1D) proved low and was virtually identical in the 2 groups. With a median follow-up time of 3 years, overall survival (PBSCs vs BM: 50% ± 5% vs 46% ± 6%, respectively; $P = .63$) and event-free survival (PBSCs vs BM: 45% ± 5% vs 44% ± 6%, respectively; $P = .59$) were comparable (Figure 1E-F). In multivariable analysis, taking into account all parameters with $P < .2$ in univariate analysis, the only significant independent risk factor for treatment failure was advanced disease status at the time of transplantation (relative risk = 2.4, 95% confidence interval, 1.5-3.8; $P = .001$). In contrast, stem cell source (PBSCs vs BM) had no effect (relative risk = 1.1, 95% confidence interval, 0.7-1.6; $P = .8$).

Our registry-based analysis provides evidence that in pediatric recipients of HLA-matched unrelated-donor transplantation with consistent antithymocyte globulin (ATG) use during conditioning, transplantation with PBSCs and BM results in comparable clinical outcomes without detectable differences in the risk of acute or, more importantly, chronic GVHD. Consistent with a recent study underscoring the role of ATG for the prevention of acute and chronic GVHD,[5] the use of ATG in 96% of our transplantation procedures compared with only 27% in the above-mentioned randomized study by Anasetti et al[4] might be one of the key factors responsible for the overall low and comparable incidence of

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Ribosomal protein deficiency causes Tp53-independent erythropoiesis failure in zebrafish

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ABSTRACT

Diamond-Blackfan anemia is an inherited genetic disease caused by mutations in ribosomal protein genes. The disease is characterized by bone marrow failure, congenital anomalies, and a severe erythroid defect. The activation of the TP53 pathway has been suggested to be critical for the pathophysiology of Diamond-Blackfan anemia. While this pathway plays a role in the morphological defects that associate with ribosomal protein loss-of-function in animal models, its role in the erythroid defects has not been clearly established. To understand the specificity of erythroid defects in Diamond-Blackfan anemia, we knocked down five RP genes (two Diamond-Blackfan anemia-associated and three non-Diamond-Blackfan anemia-associated) in zebrafish and analyzed the effects on the developmental and erythroid phenotypes in the presence and absence of Tp53. The co-inhibition of Tp53 activity rescued the morphological deformities but did not alleviate the erythroid aplasia indicating that ribosomal protein deficiency causes erythroid failure in a Tp53-independent manner. Interestingly, treatment with L-Leucine or L-Arginine, amino acids that augment mRNA translation via mTOR pathway, rescued the morphological defects and resulted in a substantial recovery of erythroid cells. Our results suggest that altered translation because of impaired ribosome function could be responsible for the morphological and erythroid defects in ribosomal protein-deficient zebrafish.

1. Introduction

Mutations in genes that encode proteins involved in ribosome biogenesis can cause specific disease conditions in humans called ribosomopathies, which are a collection of rare genetic disorders that mainly affect the bone marrow (Narla and Ebert, 2010). Diamond-Blackfan anemia (DBA) is a ribosomopathy caused by the mutation of ribosomal protein (RP) genes. DBA patients typically display a prominent tissue-specific phenotype: a loss of erythroid cells in the bone marrow that results in severe anemia. Some patients also exhibit other pleiotropic anomalies, such as growth retardation, craniofacial deformities, upper limb malformations and heart and kidney dysfunction (Lipton and Ellis, 2010). Currently, mutations have been identified in 10 RP genes (DBA-associated RPs); *RPS19* is the most frequently mutated gene in DBA patients (25% of the patients). The other DBA-associated RPs, which are less frequently mutated in patients, include *RPL5* (7%), *RPL11* (5–10%), *RPL35A* (2–4%), *RPS24* (2%), *RPS7* (1%), *RPS10* (2–6%), *RPS17* (1%), *RPS26* (2–6%) and *RPL26* (<1%) (Gazda et al., 2012; Vlachos et al., 2013). The recent identification of a large deletion in *RPL15* in a DBA patient expanded the list of DBA-associated RPs (Landowski et al., 2013). These known RP mutations now account for approximately half (~55%) of the DBA patients. However, the genes mutated in the other DBA patients remain unknown. Although it has been confirmed that ribosome biogenesis is defective in DBA, it is unclear how impaired ribosome synthesis specifically affects erythrocyte maturation.

The disruption of ribosome biogenesis evokes a nucleolar stress response, which activates the TP53 signaling pathway (Chakraborty et al., 2011). Studies in a variety of cellular and animal models have highlighted the critical role of TP53 in the clinical manifestation of DBA. Human haematopoietic progenitor cells harboring an shRNA-mediated knockdown of the *RPS19* gene display increased TP53 activity specifically in the erythroid lineage (Dutt et al., 2011). In transgenic mice with RPS19 deficiency, activated TP53 was responsible for the anemia phenotype (Jaako et al., 2011). Similarly, in zebrafish, the loss of Rpl11 and Rps29 resulted in Tp53-mediated developmental defects and impaired erythropoiesis (Danilova et al., 2011; Taylor et al., 2012). In previous studies, we demonstrated that the knockdown of RPs caused numerous common anomalies in zebrafish (Uechi et al., 2006) and that the co-inhibition

Abbreviations: DBA, Diamond-Blackfan Anemia; RP, ribosomal protein; MO, morpholino antisense oligo nucleotide; mTOR, mammalian target of rapamycin.

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of Tp53 rescued the morphological abnormalities associated with Rpl11 and Rps19 deficiency (Chakraborty et al., 2009; Torihara et al., 2011). It is generally accepted that the TP53 pathway is responsible for the morphological phenotypes that are associated with DBA. However, the role of TP53 in the erythroid phenotype of DBA has not been clearly established. The knockdown of *RPS19* in erythroid cells resulted in cell cycle arrest and proliferation defects via TP53-independent pathways (Iadevaia et al., 2010). Similarly, we have demonstrated that Rps19 deficiency in zebrafish caused erythroid defects, even in the absence of Tp53 (Torihara et al., 2011). Interestingly, in Rps7-deficient zebrafish, the simultaneous suppression of Tp53 activity resulted in incomplete rescue of the erythroid and morphological abnormalities (Duan et al., 2011).

In this study, we knocked down five RP genes (two DBA-associated and three non-DBA-associated) in zebrafish and analyzed the consequences of RP deficiency on morphological and erythroid development in the presence and absence of Tp53. Our results indicated that any RP deficiency, regardless of its role in DBA, led to erythropoietic failure in zebrafish in a Tp53-independent manner. We further demonstrated that treatment of RP-deficient embryos with amino acids that enhance translational efficiency via the mTOR pathway rescued the morphological abnormalities and the erythroid defects. Our results support the hypothesis that altered translation because of impaired ribosome function is responsible for the developmental and erythroid phenotypes that are associated with RP deficiency.

2. Materials and methods

2.1. Zebrafish maintenance

The fish were maintained in optimum conditions as per standard guidelines. The embryos were raised in E3 embryo medium at 28.5 °C. Wild-type embryos were obtained from AB lines. The *tp53* homozygous mutant line (*tp53*$^{m214k/m214k}$), which has a point mutation in the DNA-binding domain (Berghmans et al., 2005), was purchased from Zebrafish International Resource Center (ZIRC; http://zebrafish.org/zirc/home/guide.php).

2.2. Morpholino injections

Morpholinos (MOs) to knockdown RP genes were obtained from Gene Tools, LLC. For individual RPs, MOs were injected at 0.5 μg/μl using an IM-30 Electric Micro-injector (Narishige, Tokyo, Japan). In double-knockdown embryos, MOs for the RP and *tp53* were injected as a mixture at 0.5 μg/μl. The MO sequences targeting the RP genes were described previously by Uechi et al. (2006), and the p53 MO sequence was reported by Langheinrich et al. (2002).

2.3. In vitro mRNA synthesis and rescue experiments

To generate MO-resistant mRNA, five nucleotides were changed at the MO target site in the 5′UTR of *rpl35a* cDNA without altering the encoded amino acids (GenBank accession no. NM_001002487.1). The full-length *rpl35a* cDNA was PCR amplified, inserted into the pCS2+ vector and transformed in DH5α competent cells (Toyobo, Osaka, Japan). Capped *rpl35a* mRNA was generated *in vitro* from linearized pCS2+/*rpl35a* using SP6 polymerase in the mMESSAGE mMACHINE kit (Ambion, Foster City, CA, USA). Rescue experiments were performed by injecting a mixture of *rpl35a* mRNA (350 ng/μl) and *rpl35a* MO (0.5 μg/μl).

2.4. Hemoglobin staining

The density of blood cells around the cardial vein at 48 h post fertilization (hpf) was detected by hemoglobin staining using *o*-dianisidine as previously described (Detrich et al., 1995).

2.5. Amino acid and rapamycin treatment

L-Leucine (Sigma–Aldrich, St. Louis, MO, USA) was utilized at a final concentration of 100 mM as previously described (Payne et al., 2012). L-Arginine monohydrochloride (Sigma–Aldrich, St. Louis, MO, USA) was utilized at a final concentration of 50 mM. L-Alanine and L-Glycine (Wako, Osaka, Japan) were utilized at a final concentration of 100 mM, respectively. The amino acids were directly added to the E3 embryo media, and 1-day-old embryos were treated for 24 h. Rapamycin (LC Laboratories, Woburn, MA, USA) was prepared in DMSO and added to the E3 embryo media at a final concentration of 0.25 μM, 0.5 μM and 1.0 μM. To prevent pigmentation in the treated embryos, PTU was added at a final concentration of 0.2 mM.

3. Results

3.1. Developmental abnormalities in RP-deficient zebrafish

Previously, we demonstrated that the systematic knockdown of 21 RPs caused developmental defects in zebrafish and that increased Tp53 activity was responsible for the morphological abnormalities that are associated with the deficiency of two DBA-associated RPs (Rpl11 and Rps19) (Uechi et al., 2006; Chakraborty et al., 2009; Torihara et al., 2011). To determine whether Tp53 is responsible for the common morphological defects that are observed in other RP-deficient zebrafish (Uechi et al., 2006), we knocked down five RP genes, including two DBA-associated genes and three non-DBA-associated genes, and analyzed the consequences of RP depletion on embryonic development. For the DBA-associated RPs, we chose to study *rpl35a* and *rps24* because loss-of-function analyses of these two RPs have not yet been performed in animal models. For the non-DBA-associated RPs, we selected *rps3*, *rpl35* and *rplp1* because no mutations have been identified in these RP genes in DBA patients.

At 24 hpf, a stage when many organs are already recognizable, all of the RP knockdown zebrafish displayed morphological abnormalities, such as aplasia in the brain and a bent tail (Fig. 1A and B). Other anomalies commonly associated with RP knockdown embryos, such as a thin yolk sac extension, a thin body trunk and reduced body length, were also observed in the RP-deficient embryos (Fig. 1A and B). By 48 hpf, when morphogenesis is almost complete and the blood cells are in circulation, the RP knockdown embryos had significantly smaller heads and eyes, a defective heart with pericardial edema and reduced pigmentation (Fig. 1C and Supplementary Fig. 1). The control MO-injected embryos did not exhibit any morphological defects (Fig. 1C and Supplementary Fig. 2). The co-injection of MO-resistant mRNA completely rescued the developmental defects, indicating that the phenotypes specifically resulted from RP deficiency (data shown for *rpl35a*, Fig. 1A and C).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.01.006.

3.2. Rescuing the developmental abnormalities in RP-deficient zebrafish via the simultaneous loss of functional Tp53

The morphological defects in RP-deficient zebrafish are Tp53-dependent. To determine whether this was true for the five

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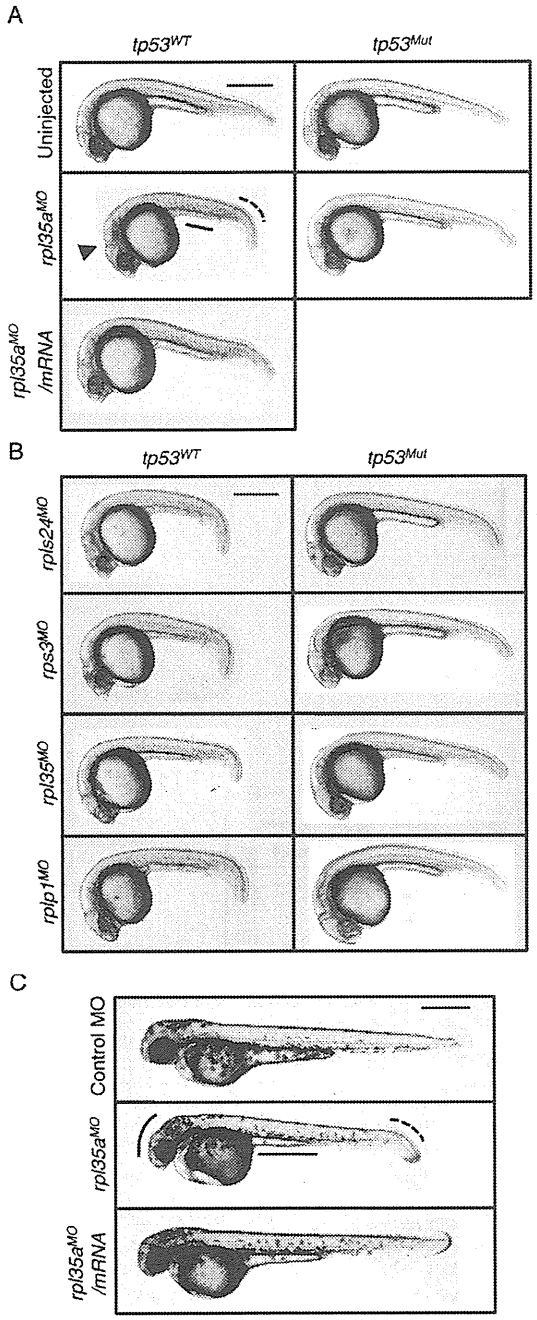

Fig. 1. Morphological abnormalities in RP-deficient zebrafish. Lateral images of embryos at 24 and 48 hpf. (A) Knockdown of *rpl35a* (*rpl35a*^{MO}) in wild-type embryos (*tp53*^{WT}) caused aplasia in the brain (indicated with a triangle), small eyes, a thin yolk extension (black solid line) and trunk, reduced body length and a bent tail (black dotted curve) at 24 hpf. The co-injection of *rpl35a* mRNA and *rpl35a* MO (*rpl35a*^{MO}/*mRNA*) or the knockdown of *rpl35a* in *tp53* mutants (*tp53*^{Mut}) completely rescued these morphological phenotypes. (B) The individual knockdown of RP genes (*rps24, rps3, rpl35 or rplp1*) resulted in similar morphological phenotypes in *tp53*^{WT}, but not *tp53*^{Mut}, embryos. (C) The co-injection of exogenous *rpl35a* mRNA rescued the morphological defects in Rpl35a-deficient zebrafish, even at later stages of development (48 hpf). Scale bar: 500 μm.

RP-deficient zebrafish studied here, we knocked down the five RPs in *tp53* homozygous mutant (*tp53*^{Mut}) embryos and observed the phenotypes at 24 hpf. As expected, none of the RP-deficient/*tp53*-mutant embryos displayed any abnormalities, indicating that the simultaneous loss of Tp53 rescued the developmental defects in the RP-deficient zebrafish (Fig. 1A and B). The double knockdown of RP and Tp53 using MOs in a wild-type background (*tp53*^{WT}) resulted in embryos that were indistinguishable from uninjected controls, further confirming the role of Tp53 in the morphological abnormalities that are associated with RP deficiency (data not shown).

3.3. The incomplete recovery of erythroid cells in RP-deficient zebrafish by the simultaneous loss of functional Tp53

The most conspicuous phenotype of DBA is the loss of erythroid cells in the bone marrow, which results in severe anemia. In previous studies, we demonstrated that the loss of Rps19 resulted in a drastic reduction of erythrocytes in zebrafish in a Tp53-independent manner (Uechi et al., 2008; Torihara et al., 2011). To determine whether the loss of Rpl35a recapitulates the erythroid phenotype of DBA in zebrafish, we performed hemoglobin staining of Rpl35a-deficient wild-type (*tp53*^{WT}) and *tp53*-mutant (*tp53*^{Mut}) embryos. The number of circulating erythrocytes was significantly reduced in the cardial vein of Rpl35a-deficient embryos compared with the uninjected controls (Fig. 2A). The simultaneous knockdown of *tp53* failed to restore the erythrocyte count in Rpl35a-deficient embryos, whereas the coinjection of MO-resistant *rpl35a* mRNA resulted in a nearly complete recovery of blood cells (Fig. 2A). The knockdown of *rpl35a* in *tp53*-mutant embryos significantly decreased the erythrocyte count compared with uninjected and control MO-injected embryos (Fig. 2A). Similarly, the knockdown of another DBA-associated RP, *rps24*, and three other non-DBA-associated RPs (*rps3*, *rpl35* and *rplp1*) in *tp53*^{Mut} embryos resulted in severe erythroid failure, as evidenced by the significant reduction in hemoglobin staining intensity in the cardial vein (Fig. 2B). The co-inhibition of these four RPs and Tp53 in *tp53*^{WT} embryos also resulted in similar erythroid defects (Fig. 2B). The co-injection of Control MO and Tp53 MO in *tp53*^{WT} embryos did not result in any erythroid defects (Fig. 2B). We further evaluated the decreased erythrocyte density in Rpl35a- and Rps24-deficient embryos compared with uninjected and mRNA-rescued embryos and categorized them into three levels: normal, moderate and severely reduced. The majority of the Rpl35a-deficient embryos (91%) exhibited a severely reduced erythrocyte density in the *tp53*-mutant background (*rpl35a*^{MO}/*tp53*^{Mut}), which was comparable to the percentage of double knockdown embryos with a severely reduced erythrocyte density (88%) in the wild-type background (*rpl35a*^{MO}/*tp53*^{MO}) (Fig. 2C). Only a small percentage of the embryos (9-12%) displayed a moderate decrease in erythrocytes in both *tp53*-mutant and the Tp53-deficient wild-type backgrounds. In contrast, the majority of the *rpl35a* mRNA-rescued embryos (88%) had a normal erythrocyte density. As expected, 100% of the Rpl35A-deficient embryos displayed severely reduced erythrocyte density in the presence of Tp53 (*rpl35a*^{MO}/*tp53*^{WT}), whereas none of the uninjected *tp53*^{WT} or *tp53*^{Mut} embryos exhibited severe or moderate decreases in erythrocyte density. Similar reductions in erythrocyte density were observed when *rps24* was knocked down in the *tp53*-mutant and the Tp53-deficient wild-type backgrounds (Fig. 2C). These results indicated that the deficiency of any RP, regardless of its role in DBA, led to erythroid failure and that the simultaneous suppression of Tp53 function did not rescue the erythroid defects in RP-deficient zebrafish.

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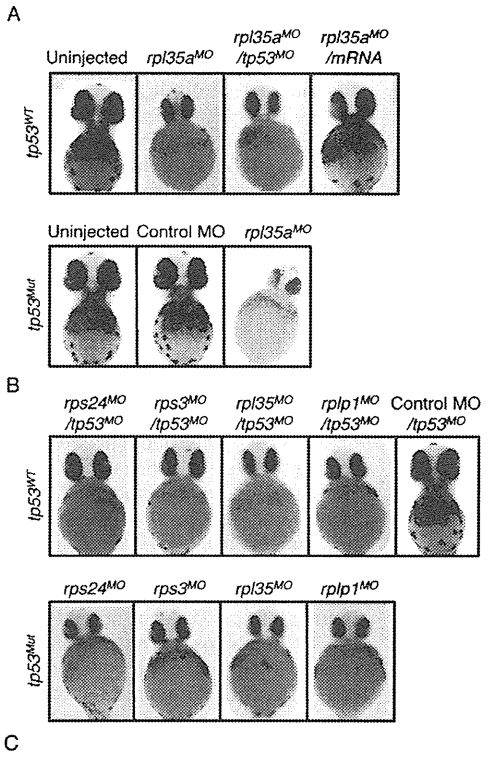

Erythrocyte density	rpl35a^MO /tp53^WT n=32	rpl35a^MO /tp53^MO n=36	rpl35a^MO /tp53^Mut n=34	rpl35a^MO /mRNA n=32	rps24^MO /tp53^WT n=27	rps24^MO /tp53^MO n=31	rps24^MO /tp53^Mut n=31	tp53^WT n=33	tp53^Mut n=34	
Normal	0	0	0	88	0	0	0	100	100	(%)
Moderate	0	12	9	12	0	6	10	0	0	
Severe	100	88	91	0	100	94	90	0	0	

Fig. 2. Erythropoiesis failure in RP-deficient zebrafish. (A-B) Hemoglobin staining of cardial veins at 48 hpf. (A) Compared with the uninjected embryos, the wild-type ($tp53^{WT}$) and *tp53* mutant ($tp53^{Mut}$) embryos injected with *rpl35a* MO ($rpl35a^{MO}$) exhibited a significant reduction in the number of hemoglobin-stained cells (orange dots). $tp53^{WT}$ embryos co-injected with *rpl35a* mRNA and *rpl35a* MO ($rpl35a^{MO}$/*mRNA*) had a nearly complete recovery of blood cells, whereas only a few blood cells were observed in double-knockdown $tp53^{WT}$ embryos ($rpl35a^{MO}/tp53^{MO}$) or in *rpl35a*-knockdown $tp53^{Mut}$ embryos ($rpl35a^{MO}/tp53^{Mut}$). Control MO-injected embryos did not exhibit any differences in the hemoglobin staining pattern compared with uninjected embryos. (B) The double-knockdown of RPs ($rps24^{MO}$, $rps3^{MO}$, $rpl35^{MO}$ or $rplp1^{MO}$) and *tp53* ($tp53^{MO}$) in $tp53^{WT}$ embryos (upper panel) or the single-knockdown in $tp53^{Mut}$ embryos (lower panel) resulted in very weak hemoglobin staining, suggesting a decrease in the number of blood cells. Control MO and tp53 MO-injected embryos exhibited a normal hemoglobin staining pattern. (C) The density of circulating erythrocytes was scored at three levels: normal, moderate and severe deficiency, relative to uninjected embryos. The percentage of embryos in each level was calculated. The highlighted values represent the most frequently observed level of the erythrocyte density in each group.

3.4. Rescuing the morphological and erythroid defects in RP-deficient zebrafish with L-Leucine or L-Arginine treatment

Certain amino acids, such as L-Leucine and L-Arginine, stimulate cell proliferation by augmenting cap-dependent mRNA translation through the mTOR-RPS6K-RPS6-EIF4EBP1 signaling pathway (Kim et al., 2013). Previously, Payne et al. (2012) demonstrated that L-Leucine improved the developmental and anemia phenotypes in Rps19- and Rps14-deficient zebrafish by activating the mammalian target of rapamycin complex 1 (mTORC1), which is an important component of the mTOR pathway. To determine whether L-Leucine or L-Arginine could alleviate the morphological and erythroid defects in Rpl35a-deficient embryos, we treated $rpl35a^{MO}/tp53^{WT}$ embryos with L-Leucine or L-Arginine for 24 h and analyzed the effects on the morphological and erythroid defects. Compared with untreated knockdown embryos, the morphological abnormalities, such as the small eyes and bent tail that are associated with Rpl35a deficiency, were almost completely reversed in L-Leucine- or L-Arginine-treated embryos (Fig. 3). We also observed a substantial recovery of the anemia phenotype in L-Leucine- or L-Arginine-treated Rpl35a-deficient embryos, as evidenced by the significant increase in hemoglobin staining in the cardial vein (Fig. 4A). Treatment with L-Leucine or L-Arginine returned the erythrocyte density to normal in 48% or 59%, respectively, of the Rpl35a-deficient embryos (Fig. 4B). To determine if the phenotypic rescue in Rpl35a-deficient embryos by L-Leucine and L-Arginine was due to the activation of the mTOR pathway, we treated the embryos with L-Alanine or L-Glycine, amino acids not known to stimulate the mTOR signaling. Treatments with L-Alanine and L-Glycine did not improve the morphological defects or anemia in Rpl35a-deficient embryos, suggesting an involvement of the mTOR pathway (Supplementary Figs. 3 and 4). To further confirm the role of mTOR, we assessed the effects of rapamycin, a specific inhibitor of the mTORC1 complex, on the phenotypic rescue of amino acid-treated Rpl35a-deficient embryos. The presence of rapamycin prevented the morphological and erythroid recovery in

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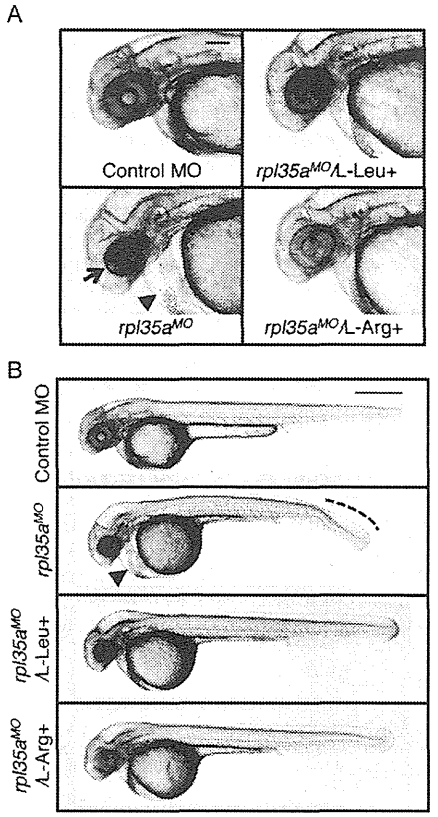
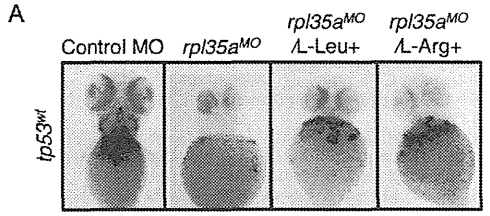


Fig. 3. Amino acid treatment of Rpl35a-deficient embryos. (A-B) Lateral images of embryos at 48 hpf showing an almost complete recovery of the small eye (arrow), pericardial edema (black triangle) and bent tail (dotted line) phenotypes in Rpl35a-deficient embryos (*rpl35a*^{MO}) after treatment with L-Leucine (*rpl35a*^{MO}/L-Leu+) or L-Arginine (*rpl35a*^{MO}/L-Arg+). Scale bar: A, 100 μm and B, 500 μm.



B

Erythrocyte density	*rpl35a*^{MO} n=24	*rpl35a*^{MO} /L-Leu+ n=23	*rpl35a*^{MO} /L-Arg+ n=22	Control MO n=23	
Normal	0	48	59	100	(%)
Moderate	0	17	14	0	
Severe	100	35	27	0	

Fig. 4. Erythrocyte recovery in Rpl35a-deficient embryos treated with amino acids. (A) Hemoglobin staining of cardial veins at day 3 post-fertilization. Compared with the control MO-injected group, Rpl35a-deficient embryos (*rpl35a*^{MO}) exhibited severe anemia, but treatment with L-Leucine (*rpl35a*^{MO}/L-Leu+) or L-Arginine (*rpl35a*^{MO}/L-Arg+) significantly reversed this phenotype. (B) The density of circulating erythrocytes was scored at three levels: normal, moderate and severe deficiency, relative to uninjected embryos. The percentage of embryos in each level was calculated. The highlighted values represent the most frequently observed level of the erythrocyte density in each group.

L-Leucine and L-Arginine treated Rpl35a-deficient embryos (Supplementary Figs. 3 and 4). Treatment with rapamycin alone did not result in any morphological or erythroid phenotypes in the wild-type embryos (Supplementary Figs. 3 and 4). These results indicated that L-Leucine or L-Arginine improved the developmental and erythroid abnormalities of RP-deficient zebrafish via activation of the mTOR pathway.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.01.006.

4. Discussion

Over the last decade, remarkable progress has been made in understanding the molecular mechanisms of DBA pathophysiology. What was initially thought to be a disease involving extra-ribosomal functions of RPS19 has now been confirmed as a disease of ribosome dysfunction. Although *RPS19* remains the most commonly mutated gene in DBA, genome-wide exome sequencing of all RP genes in a large number of DBA patients without mutations in *RPS19* has revealed mutations in 9 other RP genes (reviewed in Chakraborty and Kenmochi, 2012; Gazda et al., 2012). Recently, an array-comparative genomic hybridization analysis identified large deletions in another novel RP gene, *RPL15* (Landowski et al., 2013). Currently, 11 RPs are known to be involved in DBA, but these identified mutations only account for approximately half (~55%) of all DBA cases; the genes that are mutated in the remaining DBA cases are unknown.

Several hypotheses have been proposed for the pathophysiology of DBA (Ball, 2011), but it is unclear how mutations in ubiquitously expressed RP genes specifically affect erythropoiesis. Ribosomal malfunction triggers nucleolar stress, which results in the translocation of several RPs to the nucleoplasm where they bind to and sequester MDM2 from TP53, leading to the activation of the TP53 signalling pathway (reviewed in Chakraborty and Kenmochi, 2012). Several *in vitro* (Sieff et al., 2010; Dutt et al., 2011; Moniz et al., 2012) and *in vivo* (McGowan et al., 2008; Danilova et al., 2008; Danilova et al., 2011; Jaako et al., 2011) studies have highlighted the central role of TP53 in erythropoietic failure in DBA. However, *RPL11* and *RPL5*, two important RPs essential for ribosomal stress-mediated TP53 activation, are mutated in DBA (Gazda et al., 2008), indicating that TP53-independent pathways could also play a role in the erythroid phenotype of DBA. In this study, we observed that the individual knockdown of five different RP genes resulted in morphological abnormalities and erythroid defects in zebrafish embryos. Suppressing Tp53 activity in zebrafish embryos, either by genetic inactivation or antisense-mediated inhibition, rescued the morphological abnormalities, which is consistent with the results obtained in mouse and zebrafish models of DBA (McGowan et al., 2008; Chakraborty et al., 2009; Torihara et al., 2011), but failed to restore the erythroid cell count. Interestingly, erythroid insufficiency was a common phenotype in all the RP-deficient zebrafish, including the non-DBA-associated RPs.

We do not know whether erythroid defect is a general response to the deficiency of all the RPs, but our results suggested that the erythroid failure observed in our RP-deficient zebrafish is independent of Tp53 activity, which is in contrast to the widely accepted notion that the erythroid insufficiency in DBA is due to an activated TP53 response. It should be noted that despite the development of several cellular and animal models for DBA, the observations in these systems have not been consistent and the role of TP53 has not been clearly defined. For instance, RPS19-deficient human hematopoietic progenitor cells (Dutt et al., 2011) or *RPS19* mutated primary hematopoietic cells from DBA patients (Moniz et al., 2012) exhibited cell cycle arrest and apoptosis in the erythroid lineage,

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which were reverted upon TP53 depletion. However, loss of RPS19 resulted in cell cycle arrest and block of cell proliferation even in TP53 mutated erythroid cell lines (Iadevaia et al., 2010), suggesting that the erythroid defect upon RP deficiency is not necessarily dependent on functional TP53 alone. Similarly, in animal models of DBA, the severity of erythroid defects has been a matter of concern. RPS19 heterozygous mice displayed either a normal phenotype (Matsson et al., 2004) or mild anemia (McGowan et al., 2008; Jaako et al., 2011), whereas RPS7 heterozygous mice showed no apparent erythroid defects (Watkins-Chow et al., 2013). Although the suppression of TP53 rescued the erythroid defects in RPS19-deficient mice, it is not known if the same could be recapitulated in mouse models with severe anemic conditions as seen in DBA patients. On the other hand, in zebrafish, deficiency of DBA-associated RPs (Rps19, Rpl11, and Rps7) resulted in severe anemia (Uechi et al., 2008; Danilova et al., 2008; Duan et al., 2011; Danilova et al., 2011), which is comparable to the patient phenotype, but the role of Tp53 has been controversial. For instance, when Rps19 was knocked down in *tp53*$^{-/-}$ mutants the morphological and developmental defects were rescued (Danilova et al., 2008), but the erythroid defects were not rescued when Tp53 was co-inhibited by MO (Torihara et al., 2011). In contrast, MO-mediated tp53 depletion in *rpl11*$^{-/-}$ mutants resulted in increased erythrocyte counts compared to un-injected *rpl11*$^{-/-}$ mutants (Danilova et al., 2011). We speculate that the variation in Tp53 response could be caused by differences between homozygous mutants and knocked down animals. The maternal mRNA deposited in homozygous mutant embryos from the heterozygous parent might compensate in part for the loss of an RP. On the other hand, the antisense morpholino would be expected to knockdown any target mRNA, including the maternally derived ones. In this study, we used both *tp53* MO and *tp53* $^{-/-}$ mutants in order to avoid discrepancies that might arise due to differences in experimental approach (antisense-mediated inhibition vs. genetic inactivation) as seen in previous studies.

In this study, treatment with amino acids, such as L-Leucine or L-Arginine, rescued the anemia and morphological abnormalities in Rpl35a-deficient embryos. L-Leucine was previously reported to improve the anemia phenotype in zebrafish and mouse models of DBA (Payne et al., 2012; Jaako et al., 2012), and our data corroborate these previous observations. Studies of DBA patient-derived lymphoblasts and fibroblasts have demonstrated that haploinsufficiency of an RP leads to a reduction in global translation (Cmejlova et al., 2006; Avondo et al., 2009). Our RP-deficient zebrafish recovered from the morphological and erythroid defects when they were treated with amino acids, which stimulate cap-dependent mRNA translation via mTOR activation (Kim et al., 2013). Treatment with rapamycin, an inhibitor of mTORC1, abrogated the morphological and erythroid recovery in amino acid-treated RP-deficient embryos, further confirming the involvement of the mTOR pathway. These findings strongly indicated that the morphological and erythroid defects in RP-deficient zebrafish embryos might be due to a decrease in overall translation. The haploinsufficiency of ribosomal proteins results in defective ribosome biogenesis, which may limit the number of functionally active ribosomes or increase the abundance of defective ribosomes. A quantitative or qualitative change in ribosome production may alter the translational output, resulting in decreased overall translation or in the selective inhibition of the translation of specific mRNAs. Recently, it was reported that the loss of RPS19 and RPL11 in mouse erythroblasts impaired the translation of erythroid-specific transcripts, such as *BAG1* and *CSDE*, although the role of these proteins in erythropoiesis is not clearly defined (Horos et al., 2012).

The identification of mutations in several RP genes in DBA patients has made it clear that DBA is not a disease caused by the loss of function of a specific RP but is instead a disease caused by defects in overall ribosome production. Ribosome biogenesis defects lead to two different outcomes: a cellular stress response that involves TP53 and impaired translation that alters the translational output. In this study, the morphological abnormalities associated with RP deficiency were dependent on Tp53, but the erythroid defects were Tp53-independent. Interestingly, the morphological and erythroid abnormalities improved when the RP-deficient zebrafish were treated with amino acids, which augment mRNA translation via mTOR activation. Therefore, we hypothesized that the reduced expression of RPs decreased the translational efficiency of the ribosomes, which impacted global translation. However, the specificity of erythroid defects in DBA suggests that the effects of decreased translation may be more pronounced on particular mRNAs that are specifically expressed in erythroid cells. Identifying these mRNAs will be crucial for developing efficient diagnostic and treatment strategies for DBA.

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特集：リボソームの機能調節と疾患

II.　リボソームRNAの転写後修飾とアセンブリー
II-6　リボソーム病—リボソーム合成の異常と疾患—

剣持直哉

リボソームはすべての生物に不可欠なタンパク質の合成工場である．真核生物では，4種類のRNAと79種類のタンパク質からなる巨大な複合体を形成している．最近，ダイアモンド・ブラックファン貧血をはじめとする種々の疾患で，リボソーム合成に関わる遺伝子の変異が次々と報告され大きな関心を呼んでいる．これらはまとめてリボソーム病と呼ばれているが，広範に存在するリボソームの異常がなぜ特定の臓器や細胞にのみ影響を与えるのか，大きな謎である．p53が重要な役割を果たしていると思われるが，それだけでは説明できない．本稿では，リボソーム病の概要を解説するとともに，ダイアモンド・ブラックファン貧血を中心に発症機構の解明に向けた最新の取り組みについて紹介する．

1. はじめに

細胞内には多数のリボソームが存在する．真核生物では，4種類のRNA（rRNA）と79種類のタンパク質（RP）からなる巨大なRNA・タンパク質複合体（RNP）を形成している[1]．リボソームの生合成はこれら構成成分のほかに，多数の因子が関わる複雑な過程を経て進行する（吉川らの項を参照）．リボソーム生合成の各ステップは厳密に制御されており，その異常は核小体にストレスを引き起こしp53経路を活性化する．その結果，細胞周期の停止やアポトーシスが誘導される．また，これが疾患の原因となる場合もある[2]．当初，リボソームに生じた異常は胎生致死となり，疾患の原因にはなりえないと考えられてきた．しかし，1999年にダイアモンド・ブラックファン貧血（DBA）の患者でリボソームタンパク質S19遺伝子（*RPS19*）に変異が発見されたことを契機に，ほかの遺伝子にも次々と変異が同定された[3]．現在では，造血障害を呈するほかの疾患でもリボソーム関連因子に変異が見つかり，これらはまとめてリボソーム病（ribosomopathy）と呼ばれている[4]．本稿では，リボソーム病の最新の動向と発症機構の解明に向けた取り組みなど，筆者らのゼブラフィッシュを用いたアプローチも交えて紹介する．

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Ribosomopathies—Defective ribosome biogenesis and diseases—

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2. リボソーム病

これまでに報告のあったリボソーム病を**表1**にまとめた．前述のとおりダイアモンド・ブラックファン貧血を皮切りに，造血異常を示す種々の疾患でリボソームの関与が明らかになっている．最近では，患者のエキソーム解析により無脾症などその他の疾患でも報告がある．ここでは各々のリボソーム病について概説するとともに，p53との関連についてもふれたい．

1）造血不全，骨格形成不全，発がん性を示す疾患

ダイアモンド・ブラックファン貧血

ダイアモンド・ブラックファン貧血（Diamond-Blackfan anemia：DBA）は，赤血球造血のみが障害される先天性の造血不全症である．骨髄では赤芽球系の細胞がほとんどみられず，発がんリスクも高い．また，低体重や手指の奇形を伴うこともある．これまでに11種類のRP遺伝子，