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Cross talk between TP53 and c-Myc in the pathophysiology of Diamond-Blackfan anemia: Evidence from RPL11-deficient *in vivo* and *in vitro* models

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ABSTRACT

Mutations in genes encoding ribosomal proteins have been identified in Diamond-Blackfan anemia (DBA), a rare genetic disorder that presents with a prominent erythroid phenotype. TP53 has been implicated in the pathophysiology of DBA with ribosomal protein (RP) L11 playing a crucial role in the TP53 response. Interestingly, RPL11 also controls the transcriptional activity of c-Myc, an oncoprotein that positively regulates ribosome biogenesis. In the present study, we analyzed the consequences of *rpl11* depletion on erythropoiesis and ribosome biogenesis in zebrafish. As expected, Rpl11-deficient zebrafish exhibited defects in ribosome biogenesis and an anemia phenotype. However, co-inhibition of Tp53 did not alleviate the erythroid aplasia in these fish. Next, we explored the role of c-Myc in RPL11-deficient cellular and animal models. c-Myc and its target nucleolar proteins showed upregulation and increased localization in the head region of Rpl11-deficient zebrafish, where the morphological abnormalities and *tp53* expression were more pronounced. Interestingly, in blood cells derived from DBA patients with mutations in *RPL11*, the biogenesis of ribosomes was defective, but the expression level of c-Myc and its target nucleolar proteins was unchanged. The results suggest a model whereby RPL11 deficiency activates the synthesis of c-Myc target nucleolar proteins, which subsequently triggers a p53 response. These results further demonstrate that the induction of Tp53 mediates the morphological, but not erythroid, defects associated with RPL11 deficiency.

1. Introduction

Diamond Blackfan anemia (DBA; OMIM #105650) is a rare inherited bone marrow failure syndrome, which presents as erythroid aplasia, macrocytic anemia, and selective erythroid precursor deficiencies in the bone marrow [1]. The symptoms of DBA generally appear during infancy and are typically associated with a wide range of congenital anomalies, including short stature, thumb deformities, cleft lip/palate, cardiac and genitourinary defects [2]. This disease is also associated with an increased risk of cancer [3]. The majority of DBA patients have autosomal dominant germline mutations or deletions in ribosomal protein (RP) genes. To date, 18 RP genes have been implicated in DBA [4]. Recently, mutations in two X-linked genes (*GATA1* and *TSR2*) have been identified in a few patients [5,6]. Although these genes account for 65% of DBA cases, the genes mutated in the remaining 35% of the patients are as yet unknown.

DBA is often considered a classical example of "ribosomopathies," a group of rare genetic conditions in humans reflecting

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defects in ribosome biogenesis and function [7]. Deficiency of DBA-type RPs impairs the processing of pre-rRNAs and maturation of ribosomal subunits [2,8,9]. In fact, the analysis of rRNA ratios using capillary electrophoresis has been proposed as one of the criteria for the initial diagnosis of DBA [10]. However, although considered a ribosomopathy, the hallmark of DBA is a prominent tissue-specific phenotype involving the erythroid lineage. The tissue specificity associated with DBA is intriguing considering the fact that ribosomes are ubiquitous, and any defects in ribosomes are expected to show up in all cell types. Over the last decade, several hypotheses have been proposed to explain the molecular mechanisms of DBA [11]. Nevertheless, the TP53-signaling pathway has been implicated as the major contributor to the pathophysiology of DBA based on evidence that impaired ribosome biogenesis evokes nucleolar stress, and this stress is sensed by TP53 [12]. Several ribosomal proteins (RP) have been implicated in the nucleolar stress-mediated TP53 responses through interactions with murine double minute 2 (MDM2), the negative regulator of TP53 [13]. Among the many RPs identified, ribosomal protein (RP) L11 (uL5 in a new naming system) and RPL5 (uL18) are the most important proteins [14]. Indeed, a number of *in vitro* and *in vivo* studies have shown that the TP53-mediated cell cycle arrest and apoptosis in DBA models are RPL11 dependent [15,16].

Apart from its role in TP53 regulation, RPL11 is involved in the negative regulation of c-Myc, a transcription factor that plays a crucial role in ribosome biogenesis within the nucleolus [17]. RPL11 directly binds to the MB II domain of c-Myc protein and inhibits the recruitment of its coactivator TRRAP to the promoter region of c-Myc target genes, thereby repressing its transcriptional activity [18]. RPL11 controls *MYC* mRNA levels through miRNA-mediated repression by recruiting miR-24 to the 3′ untranslated region of c-Myc [19].

In a previous study, we showed that the loss of DBA-associated RPs, including RPL11, activates the Tp53 pathway in zebrafish [20–22]. We also showed that the co-inhibition of Tp53 rescues the morphological phenotype associated with RP deficiency but does not alleviate the erythroid aplasia observed in these fishes [20–22]. Here, we analyzed the effects of RPL11 deficiency on erythropoiesis and ribosome biogenesis using a combination of cellular and animal models of DBA and investigated the role of c-Myc in the pathophysiology of DBA.

2. Materials and methods

2.1. Zebrafish maintenance, morpholino injections and hemoglobin staining

Adult zebrafish (*Danio rerio*) were raised and maintained according to standard guidelines [23]. Wild-type embryos were obtained from AB lines. The embryos were raised in E3 medium at 28.5°C. The *rpl11* mutant zebrafish ($rpl11^{hi3820bTg}$), which harbors a viral insertion in the first intron of *rpl11* gene [24] and the *tp53* homozygous mutant line ($tp53^{m214k/m214k}$), which has a point mutation in the DNA-binding domain [25], were purchased from Zebrafish International Resource Center (ZIRC). All experimental procedures were performed in accordance with relevant guidelines and regulations and approved by the University of Miyazaki. Morpholinos (MOs) were obtained from Gene Tools, LLC. The MO sequences targeting the *rpl11* gene were obtained according to Chakraborty et al. [20], and the p53 MO sequence was obtained according to Langheinrich et al. [26]. The MOs were injected at 0.5 μg/μL using an IM-30 Electric Micro-injector (Narishige, Tokyo, Japan). Hemoglobin staining was performed using *o*-dianisidine (Sigma) as previously described [27].

2.2. Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from a pool of 25–30 embryos using the RNeasy Mini Kit (Qiagen, Germany). cDNA was synthesized from 2 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Real-time PCR was performed using Power SYBR Green Master Mix (Applied Biosystems, USA) and the ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA). Changes in the transcript levels of c-Myc and nucleolar proteins were expressed as fold-changes relative to wild-type controls and normalized to *elF1a* expression using the ΔC_t method. The p value was calculated using a *t*-test, and a value of <0.05 was considered statistically significant.

2.3. Sucrose gradient fractionation of cytoplasmic ribosomes

The ribosomes were analyzed on sucrose gradients as previously described [9]. Briefly, LCLs were first treated with 100 μg/mL cycloheximide (Sigma) for 10 min, followed by mechanical disruption using a Dounce homogenizer in buffer A containing 0.5 mM dithiothreitol. After low-speed centrifugation (4°C; 1000 *g*; 10 min), the upper cytoplasmic fraction was collected and 1 mg of total protein from this cytoplasmic fraction was loaded onto a 10–50% sucrose gradient. The fractionation was performed at 160,000 *g* for 105 min at 4°C.

2.4. Analysis of rRNA by northern blot

Total RNA isolated from control and patient-type LCLs were ethanol precipitated and the RNA pellets were dissolved in formamide at a concentration of 1 mg/mL. For electrophoresis, 3 μg of total RNA/well/sample was separated on a 1.2% agarose gel containing 1.2% formaldehyde and 1X Tri/Tri Buffer (30 mM triethanolamine and 30 mM tricine, pH 7.9), which were subsequently transferred onto a Hybond N+ nylon membrane (GE Healthcare, Orsay, France) and crosslinked under ultraviolet light. The cross-linked membrane was prehybridized in 6X saline-sodium citrate (SSC), 5 Denhardt's solution, 0.5% SDS and 0.9 mg/mL tRNA at 45°C for 1 h, followed by the addition of the 5′-radiolabeled (γ^{32}-ATP) oligonucleotide probe and overnight incubation at 45°C. Post hybridization, the membranes were washed in saline-sodium citrate buffer (SSC), sodium dodecyl sulfate (SDS), and exposed for 24–48 h. Signal acquisition was performed using the FLA2000 PhosporImager (Fuji, Stamford, CT), and quantification of the signal intensity was performed with ImageGauge software.

3. Results

3.1. rpl11 mutant zebrafish display developmental anomalies, anemia, and a defective polysome profile

The Rpl11-deficient zebrafish contains a retroviral insertion in the first intron, which interferes with mRNA splicing, resulting in reduced protein expression [24]. Unlike DBA patients, the heterozygous mutants did not show any phenotype and were indistinguishable from their wild-type siblings, most likely reflecting compensation by the wild-type allele. However, the homozygous mutants ($rpl11^{Mut}$) were easily distinguishable based on the overall reduction in their body length. At 26 h post fertilization (hpf), the $rpl11^{Mut}$ zebrafish showed developmental anomalies, including microcephaly, microphthalmia, and hypopigmentation (Fig. 1A). Although circulation was not affected in these embryos, there was a significant reduction in the number of circulatory erythroid cells at 48 hpf, resulting in severe anemia (Fig. 1B). By day three, the consequences of *rpl11* depletion became more severe, with evidence of

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