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Ribosome biogenesis protein Urb2 regulates hematopoietic stem cells development via P53 pathway in zebrafish

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ABSTRACT

Ribosome biogenesis is a significant process in cells. Dysfunction in this process will result in the defects of protein synthesis and consequently cause the development of specific diseases called ribosomopathies. Mutations in ribosome biogenesis protein Rps19, Rpl5, or Rpl11 can lead to hematopoietic defects in human, thus triggering the disease Diamond Blackfan anemia. However, the regulatory mechanisms of ribosome biogenesis in hematopoiesis remain incompletely understood. In this study, we describe a zebrafish mutant *cq42*, which carries a nonsense mutation in the gene that encodes ribosome biogenesis 2 homolog (Urb2). Urb2 is strongly expressed in the caudal hematopoietic tissue (CHT) during hematopoietic stem cells (HSCs) expanding. Molecular characterization of $urb2^{cq42}$ larvae suggest that *urb2* deficiency notably decrease the population of HSCs in CHT and early T cells in thymus. Further analysis shows that compromised cell proliferation and superfluous apoptosis are observed in the CHT of $urb2^{cq42}$ mutant. P53 pathway is upregulated in the $urb2^{cq42}$ larvae and loss-of-function of P53 can fully rescue the hematopoietic defects in $urb2^{cq42}$ mutant. These data demonstrate that *urb2* is essential for HSCs development through the regulation of P53 pathway.

1. Introduction

Hematopoietic stem cells (HSCs) are those multipotent cells that have the potential to self-renew and differentiate into all blood lineages [1]. HSCs play indispensable roles in maintaining the physiological homeostasis of blood system throughout the lifetime. HSCs transplantation is restrictively used to treat hematological diseases for its shortage of sources. Thus, for the sake of producing transplantable HSCs, the molecular mechanisms of HSCs development need to be fully understood.

From the last two decades, zebrafish (*Danio rerio*) has developed to an ideal model to study hematopoiesis. The key genes and signaling pathways that regulate hematopoiesis are highly conserved in zebrafish and mammals, and zebrafish is widely used for its particular advantages to other models [1,2]. In zebrafish, HSCs specialize from aorta-gonad mesonephros at 24 hpf, indicating the initiation of definitive hematopoiesis. Then the earliest HSCs are generated from the dorsal arota through endothelial hematopoietic transition at about 30 hpf [3,4]. Subsequently, HSCs migrate to caudal hematopoietic tissue through blood circulation. At about 96 hpf, HSCs begin to populate thymus and kidney [5,6]. During the HSCs developmental process, many genes and pathways have been reported to be essential at different stages, such as Wnt, Notch, Bmp, Vegf, Hedgehog [1]. However, the list is far from complete. Thus, the forward genetic screen is capable of identifying more undetected genes that involved in hematopoiesis.

Ribosomes are essential machines for protein synthesis, and the biogenesis of ribosome is a highly conserved process in different species [7]. Ribosome biogenesis is crucial to cellular adaptation, growth, and proliferation. Disruption of the process will result in many improper genetic programs, thus triggering some specific diseases such as Diamond Blackfan anemia (DBA). Patients with DBA show hematopoietic defects during infancy or neonatal period [8]. There are approximately 80 ribosomal proteins and 170 associated proteins required during ribosome biogenesis [9]. Up to now, many papers have reported the significant roles of these proteins

Abbreviations: Urb2, ribosome biogenesis 2 homolog; HSCs, hematopoietic stem cells; CHT, caudal hematopoietic tissue; SSLP, simple sequence length polymorphic; hpf, hours post fertilization; WISH, whole mount *in situ* hybridization; WT, wild-type.

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in vitro or *in vivo*. Depletion of individual ribosomal proteins in Hela cells disrupts rRNA processing and the maturation of ribosome subunits [10]. In mouse, *rpl22* mutation causes the arrest of αβ T cell progenitors and B lymphocytes by upregulating P53 pathway [11]. In zebrafish, loss of *rpl11* will lead to the brain malformation and hematopoietic defects through P53-dependent apoptotic or cell cycle arrest responses [12,13]. Besides, the mutations of *rps19*, *rps29*, *dkc1* or *nola1* can all cause the defects of hematopoiesis through abnormal P53 pathway activation in zebrafish [14–16]. Therefore, ribosome biogenesis plays significant roles in regulating cell proliferation, apoptosis, and differentiation, thus important for organs development and embryonic growth.

Urb2 is an essential gene that encodes a comparatively large protein (135.2 kDa) in yeast, and the protein localizes in the nucleolus [17]. Urb2 protein is necessary for 60S ribosomal subunit biogenesis, and mutation or depletion of *urb2* will lead to the disruption of ribosomal subunits and rRNAs [17]. Previous research have reported that Urb1, a component of pre-60S ribosomal subunits, can regulate digestive organs development by acting as a downstream modulator of mTOR complex 1 in zebrafish [18]. However, the roles of Urb2 in embryogenesis remain unclear. Here, we report that, in zebrafish, a genetic mutation of *urb2* leads to the impairment of HSCs development, and the hematopoietic defects can be fully rescued by inhibiting P53 function. Taken together, these results facilitate our understanding on the role of Urb2 in hematopoiesis through the regulation of P53 signaling in zebrafish.

2. Materials and methods

2.1. Zebrafish strains

Zebrafish of the AB genetic background, SJD genetic mapping strain, $urb2^{cq42}$ mutant line, $urb2^{cq43}$ mutant line, $p53^{M214K}$ mutant line, *Tg(cmyb:GFP)* and *Tg(runx1:GFP)* transgenic lines were raised and maintained under standard laboratory conditions [19,20]. To inhibit pigmentation, embryos were treated with 0.003% PTU (Sigma, USA) from 24 hpf.

2.2. Genetic mapping, cloning of $urb2^{cq42}$

Heterozygous *cq42* were outcrossed with the polymorphic line SJD to generate a mapping population. Genetic mapping was performed as previously described [18,19,21]. Five hundred and eighty-four meiosis recombinants had been collected and then performed to the genetic mapping. By analysis of these embryos, the *cq42* mutation locus was narrowed to twelve genes. The coding sequences of the twelve genes were examined by cloning and sequencing. Finally, the genotype of *cq42* was identified to a nonsense mutation in the gene *urb2*. The two polymorphic markers designed by our lab consist of the primers as follows: L13m20: forward primer: 5′-AGGGATGAAATAGGTGCCC-3′, reverse primer: 5′-CCAGCCTTGCTGTAATGTTC-3'; L13m10: forward primer: 5′-GAGTCATTTATAGGCCTGAC-3′, reverse primer: 5′-TAGGACACAAGGCATGTCC-3'.

2.3. Generated cq43 using CRISPR/Cas9 system

Mutant *cq43* was generated by targeting the seventh exon of *urb2* with CRISPR/Cas9 technology. The process was performed as previously described [22]. The *urb2* target sequence was depicted in Fig. 2D, and the target region was amplified by PCR using the following primers: forward primer: 5′- CACAGTGAATGCATTAGTTGC-3'; reverse primer: 5′- GTCATTCTGTGTTTGCTAACAG-3'.

2.4. In situ hybridization

Whole mount *in situ* hybridization (WISH) and fluorescent *in situ* hybridization were performed as previously described using the following probes: *cmyb*, *rag1*, *urb2*, *p53*, *mdm2*, *p21*, *ccng1* [20,23]. The WISH images were captured by SteREO Discovery V20 microscope (Carl Zeiss, Jena, Germany). The fluorescent *in situ* hybridization pictures were captured by LSM780 confocal microscope (Carl Zeiss).

2.5. EdU cell proliferation assay combined with antibody staining

The EdU assay was applied for S-phase labeling according to the manufacturer's instructions (Click-iT Kit; Invitrogen). Embryos were injected with EdU mixture containing 0.2 mM EdU, 2% DMSO and 4% phenol red at 60 hpf, then incubated at 28.5 °C incubator for 30 min. After being fixed with 4% PFA at 4 °C overnight, embryos were subjected to EdU assay. When EdU assay was finished, whole mount antibody staining was performed as previously described using antibody against GFP (1:500; Life Technologies) [20,24]. Images were captured by LSM780 confocal microscope (Carl Zeiss).

2.6. TUNEL assay combined with antibody staining

The TUNEL assay was applied for detecting apoptotic fragments as described (In Situ Cell Death Detection Kit TMR red; Roche) [19]. When TUNEL assay was finished, the antibody staining and final images capturing were performed similar to EdU assay.

2.7. Morpholino injection

The *p53* morpholino (5′-GCGCCATTGCTTTGCAAGAATTG-3′) was obtained from Gene Tools (USA) [25]. Embryos were injected with 4 ng *p53* morpholino at 1–4 cell stage.

3. Results

3.1. HSCs development is defective in cq42 mutant

To determine the essential factors involved in the definitive hematopoiesis of zebrafish, we performed an ENU-based genetic screen to identify the mutants with hematopoietic defects. By examining the expression of *cmyb*, a marker of HSCs, we isolated a HSCs-deficient zebrafish mutant *cq42*. The early HSCs emergence in *cq42* mutant was quite normal compared to wild-type (WT) from 36 hpf to 48 hpf. However, HSCs were remarkably reduced in the CHT region of *cq42* mutant since 60 hpf (Fig. 1A and B), suggesting the defect of HSCs expansion. Early T cell marker *rag1* was also examined at 96 hpf, *cq42* embryos showed little *rag1* expression in thymus (Fig. 1C), indicating that the migration of HSCs to thymus was disrupted. Under the *Tg(cmyb:GFP)* transgenic line background, the *cq42* mutant also displayed reduced HSCs at 72 hpf (Fig. 1D). The pictures in bright field showed that there was no obvious body defect in *cq42* mutant except slightly smaller eyes (Fig. 1E). These results suggest that the definitive hematopoiesis is defective in *cq42* mutant.

3.2. The mutation of cq42 mutant lies in urb2

To identify the gene responsible for *cq42* mutant phenotype, we performed a genome mapping of *cq42*. Heterozygous *cq42* mutant line fish (AB strain) were crossed with wild-type SJD strain fish to generate the F1 generation. Then we identified the F1 generation, and those fish with heterozygous *cq42* background were in-crossed to get a mapping population. The mutant allele *cq42* was

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