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Noncanonical function of threonyl-tRNA synthetase regulates vascular development in zebrafish

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ABSTRACT

The canonical functions of Aminoacyl-tRNA synthetases (AARSs) are indispensable for protein synthesis. However, recent evidence indicates that some AARSs possess additional biological functions (noncanonical functions) related to immune responses and vascular development. Here, we identified a zebrafish *cq16* mutant presenting the disorganized vessels with abnormal branching of the established intersegmental vessels (ISVs) as well as aberrant patterning of the brain vascular network after 50 h post fertilization. The *cq16* mutant gene is responsible for encoding threonyl-tRNA synthetase (*tars*) with a missense mutation. The abnormal branching of ISVs was caused by the increased expression of vascular endothelial growth factor A (*vegfa*) in $tars^{cq16}$ mutant. Inhibition of Vegf signaling suppresses the abnormal vascular branching observed in $tars^{cq16}$ mutant. Furthermore, injection of human *TARS* mRNA potently reduced the vascular aberrant branching in $tars^{cq16}$ mutant, indicating a conserved function of *tars* in regulating angiogenesis between zebrafish and human. Therefore, we conclude that noncanonical function of *tars* regulates vascular development presumably by modulating the expression of *vegfa*.

1. Introduction

During embryonic development, endothelial cells assemble the tubular network of blood vessels that transport gases, nutrients, hormones, and metabolites throughout the vertebrate body. The network of blood vessels is one of the earliest structures to develop in a vertebrate embryo. It plays important roles in regulating tissue homeostasis and would healing and is involved in the pathology of numerous diseases including inflammation and cancer [1]. In recent years, zebrafish has become an important vertebrate model for studying vascular development. Zebrafish has a closed circulatory system and the molecular mechanisms controlling angiogenesis are highly conserved between zebrafish and mammals. As a model, zebrafish provide a unique advantage in the study of vascular development *in vivo*. Zebrafish is suitable for large scale forward genetic analysis, which makes it advantageous for screening vascular defect mutants to identify new genes that regulate vascular development. Additionally, transparent zebrafish embryo expressing fluorescent protein under the control of *flk1* or *fli1a* promoter allowed us to visualize and analyze the vascular development.

Although the vascular system is complex, almost all blood vessels arise by the sprouting of new capillaries from pre-existing vessels, a process termed angiogenesis. In the past 20 years, the molecular mechanisms controlling angiogenesis have attracted much attention. Vascular endothelial growth factor (Vegf) signaling pathway plays central roles in vascular formation and function in vertebrates [2]. Vegf signaling mainly includes four kinds of ligands and corresponding receptors, and the master regulator of new blood vessel sprouting is *Vegfa* [2,3]. *Vegfa* combines its tyrosine kinase receptor Vegfr2 to activate a variety of downstream signaling pathways. It also controls several processes in endothelial cells, such as proliferation, migration and survival. The change of the *Vegfa* expression has a direct effect on angiogenesis. Hence, Heterozygous $Vegfa^{+/-}$ mice exhibit severe vascular defects [4]. Similarly, deficient vascular assembly is observed in Vegfr2-null mice [5]. The expression of *Vegfa* is mainly regulated by the hypoxia. The transcriptional factors that were reported to regulate

Abbreviations: AARSs, Aminoacyl-tRNA synthetases; ISVs, intersegmental vessels; *tars*, threonyl-tRNA synthetase; *sars*, seryl-tRNA synthetase; hpf, hours post fertilization; *Vegfa*, Vascular endothelial growth factor A; HUVECs, human umbilical vein endothelial cells.

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Vegfa expression consist of HIF, stat3, Sp1/Sp3, AP etc. [6]. Thus, identification of new transcriptional factors for regulating *Vegfa* expression has an important role in revealing the molecular mechanisms regulating angiogenesis.

Aminoacyl-tRNA synthetases (AARSs) specially attach amino acids to their corresponding tRNA adaptors in an essential reaction of protein synthesis. In addition to their aminoacylation activities (canonical activity), recent evidence indicates that AARSs possess additional biological functions (noncanonical activities), including the regulation of angiogenesis, translation, inflammatory response and apoptosis [7]. For example, tyrosyl-tRNA synthetase (YARS) can stimulate endothelial cell proliferation and migration and regulate inflammatory responses [8,9]; tryptophanyl-tRNA synthetase (WARS) apparently binds VE-cadherin to inhibit angiogenesis [10]; glutamyl-prolyl-tRNA synthetase (EPRS) as an intergral component of the GAIT complexes inhibits angiogenesis through the translational silencing of *Vegfa* [11]. Of greater interest is that mutations in zebrafish *sars* gene encoding seryl-tRNA synthetase cause abnormal vascular sprouting in trunk and head [12,13]. Although YARS, WARS and EPRS can regulate angiogenesis in cell culture, only *sars* is reported to regulate vascular development *in vivo*. It is not fully understood whether other AARSs contribute to the establishment of vascular patterning in vertebrates.

In this study, we identified a mutation in *tars* leading to ISV abundant sprouting in zebrafish and demonstrated that the noncanonical function of *tars* is involved in the regulation of vascular patterning by mediating Vegf signaling.

2. Materials and methods

2.1. Zebrafish strains

The Zebrafish (*Danio rerio*) of the AB genetic background *Tg(flk1:GFP)* transgenic line and *cq16* mutant line were raised and maintained under standard laboratory conditions according to institutional animal care and use committee protocols.

2.2. Genetic mapping

The *cq16* locus was defined by genotyping of *cq16* mutant embryos using simple sequence length polymorphic (SSLP) markers on chromosome 5. We finally identified a missense mutation in the zebrafish *tars* gene at 1505 bp (substitution from A to G), resulting in a glutamine converted to a Arginine at amino acid 502. To perform genotyping of the $tars^{cq16}$ mutation, the *tars* locus was amplified from the isolated genomic DNA by PCR using the following primers: *tars* sense, 5-GTCTTTTTCTGTGGTACAG-3; *tars* antisense, 5-GTTTCACTGAGTAAACACACC-3.

2.3. Microinjection of synthetic mRNAs

Total RNA was extracted using Trizol (Life Technologies) and reverse transcribed to cDNA using Omniscript reverse transcriptase kit (QIAGEN). Zebrafish *tars*, *sars* and human *TARS* cds fragment were amplificated and sub-cloned into the *PCS2+*, the mRNA expression vector. Capped mRNAs were synthesized by using the mMESSAGE mMACHINE (Ambion). Synthetic mRNAs were injected into the blastomere of 1- to 2-cell-stage embryos.

2.4. Treatment of vegfr inhibitor

The $tars^{cq16}$ mutant embryos were treated with Vegfr inhibitor SU5416 (0.8 μmol/L, Sigma) from 36 to 60 hpf, or 0.1% DMSO as control.

2.5. Whole-Mount in Situ Hybridization and quantitative real-time PCR

In situ hybridization and quantitative real-time PCR was performed as previously described [14]. The primers used for amplification were as previously described [12]. The synthetic probes were as follows: *tars* primers, 5-TGTGTGCCATTGAATAAGGA-3 and 5-CACCTTCATTATCAAGATAC-3.

2.6. Cells culture and immunostaining

Human umbilical vein endothelial cells (HUVECs, ALLCELLS) were raised according to standard protocol. Cell immunostaining was performed as previously described [15] using antibodies against TARS (1:100; Santa Cruz Biotechnology), green fluorescent protein (1:500; Santa Cruz Biotechnology, Santa Cruz, CA). Antibody stained tissues were imaged using ZEN2010 software equipped on an LSM780 confocal microscope (Carl Zeiss).

2.7. CRISPR/cas9

The CRISPR/Cas9 was performed as described [16,17] and the *tars* target site sequence in Fig. 2G.

3. Results

3.1. Cq16 mutant exhibits abnormal vascular sprouting in trunk and head

In a recent N-ethyl N-nitrosourea mutagenesis screen, we identified an embryonic lethal mutant *cq16* that had defects in vascular patterning. By observing *Tg(flk1: GFP)* expression, we noticed no difference between wild type (WT) and *cq16* mutant at 40 hpf (Fig. 1A and B). However, by 50 hpf the *cq16* mutant started to exhibit ectopic ISV branching adjacent to the neural tube in the trunk (Fig. 1C and D). Subsequently, the abnormal ISV branching became more and more severe in the dorsal side of the horizontal myoseptum (Fig. 1E and F). Furthermore, we observed a pronounced dilatation of the aortic arch vasculature (Fig. 1G and H) and aberrant branching of the brain vascular network at 80 hpf (Fig. 1I and J). In bright filed, *cq16* mutant has a smaller head and thinner trunk than WT at 72 hpf (Fig. 1K). Although initially unaffected, circulation gradually diminishes and stops at 5 dpf. These results indicate that *cq16* is a vascular regulator.

3.2. The gene responsible for cq16 encodes threonyl-tRNA synthetase with a missense mutation

The *cq16* mutant phenotype is similar to *sars* mutant [12,13] except that the abnormal ISV branching in *cq16* mutant appears about ten hours earlier than that in *sars* mutant. We sequenced the *sars* mRNA in *cq16* mutant but there was no difference between WT and mutant, suggesting that the gene responsible for *cq16* is not *sars*. To identify the gene responsible for this mutant, we performed the genome mapping of *cq16*. Finally, we found that there existed a A-to-G transition at 1505 bp in the *tars* gene of *cq16* mutant (Fig. 2A and B), which converted a glutamine to a arginine at amino acid 502 (Fig. 2C). Hence, the gene responsible for *cq16* is *tars* with a missense mutation.

We examined the expression of *tars* during embryo development by whole-mount *in situ* hybridization. The *tars* transcripts were maternally supplied, as evidenced by its expression in 1-cell stage (Fig. 2D), indicating that maternal *tars* may redeem the lack of functional zygotic *tars* in the progression of initial embryogenesis in $tars^{cq16}$ mutants. In the following days, *tars* was expressed