Disruption of integrin α4 in zebrafish leads to cephalic hemorrhage during development

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Integrins, transmembrane molecules that facilitate cell-to-cell and cell-to-extracellular matrix interactions, are heterodimers that consist of α- and β-subunit. The integrin α4 gene (*itgα4*) is expressed in various type of cells and tissues. Its biochemical functions and physiological roles have been revealed using cultured cell assays. In contrast, the primary effect caused by *itgα4* deletion on vertebrate development is poorly understood, because knockout mice exhibit multiple defects that can lead to embryonic lethality in the uterus. Zebrafish are a convenient vertebrate model to investigate morphogenesis during embryogenesis, because of their external fertilization and subsequent development outside the female’s body. Here, we generated a zebrafish mutant line named *itgα4 ko108* using the CRISPR/Cas9 genome editing system; the mutant genome harbored an approximately 2.0-kb deletion in the *itgα4* locus. A truncated transcript was detected in *itgα4* (+/−) or (−/−) fish but not in (+/+). The mutant transcript was hypothesized to encode a truncated Itgα4 protein due to a premature stop codon. *itgα4* (−/−) embryos obtained from the mating of heterozygous parents exhibited no apparent phenotype during development at 24 hours post-fertilization (hpf). However, approximately half of them exhibited cephalic hemorrhage at 48 hpf. The incidence ratio was significantly higher than that in (+/+ or (+/−) embryos. Embryonic hemorrhage has also been reported previously in Itgα4 knockout mice. In contrast, embryonic lethality with the other defects reported in the knockout mice was not observed in our zebrafish model. Therefore, the mutant line *itgα4 ko108* should be a useful model to investigate a physiological function for Itgα4 in the blood circulation system.

Key words: genome editing, hemorrhage, integrin α4, zebrafish

Integrin α4 (ITGα4) is the α-subunit of the integrin heterodimer and is known to be associated with ITGβ1 and β7 in mammals (Takada et al., 2007). Previous studies reported that Itgα4 knockout mice exhibit embryonic lethality due to placental or cardiovascular defects, including hemorrhage (Yang et al., 1995; Grazioli et al., 2006). However, owing in part to this, the primary role of ITGα4 in vertebrate development remains poorly understood. Therefore, we focused on zebrafish (*Danio rerio*) as a convenient vertebrate model to investigate the function of Itgα4 during embryonic development. The zebrafish is one of the traditional model organisms in the field of developmental biology because its eggs are fertilized outside the female’s body and the transparent embryo grows rapidly (Kimmel et al., 1995). Therefore, it is a good alternative vertebrate model species to observe embryonic development.

An *itgα4* orthologous gene has been identified in the zebrafish genome (Ensembl Gene ID: ENSDARG00000103056). The transcript was detected in multiple tissues such as around the eyes, cerebellum, somite, notochord and hematopoietic tissues of the zebrafish embryo (Fig. 1A; see also Karpanen et al., 2017). To delete *itgα4* function in zebrafish, we used the CRISPR/Cas9-based genome editing system (Tokumasu et al., 2016). Two single guide RNAs were designed to delete an approximately 2.0-kb sequence between exons 4 and 7 of the *itgα4* locus (Fig. 1B). To determine the genotype of the *itgα4* mutant, a specific primer pair was designed to amplify 2,610 bp including the scheduled deletion region. As a result, a mutant line with a 2,014-bp deletion in the target region was generated (Fig. 1C). Sequencing analysis revealed that exons 4 and 7 are fused on the genome, and reverse transcription (RT)-
PCR indicated that a shorter transcript is synthesized from the mutated \textit{itg}α4 allele than from the wild-type allele (Fig. 1D). These results suggested that the \textit{Itg}α4 protein synthesized from the mutant allele is truncated by premature termination (Fig. 1E). We submitted this mutant line to the Zebrafish Information Network as \textit{itg}α4 \textit{ko}108 (https://zfin.org/ZDB-ALT-190711-5).

Next, we investigated developmental phenotypes in the \textit{itg}α4 mutant zebrafish. There were no apparent defects in the \textit{itg}α4 \textit{−/−} embryos at 24 hours post-fertilization (hpf) compared with the (+/+) or (+/−) embryos. Subsequently, at 48 hpf, approximately half of the \textit{itg}α4 \textit{−/−} embryos exhibited a hemorrhage in the head and/or other anterior regions including around the eyes or aortic arches, with a depletion of circulating erythrocytes, while the (+/+) embryos did not (Fig. 1F). One tenth of the \textit{itg}α4 \textit{+/−} embryos also exhibited cephalic hemorrhage, although this incidence rate was significantly lower than that in the \textit{−/−} embryos. The hemorrhagic phenotype was not observed in the trunk region. Furthermore, there were no visible abnormalities in other morphogenesis including heart formation and beating. These results suggested that the primary phenotype in the \textit{itg}α4-deficient zebrafish was a cephalic hemorrhage caused by blood vessel abnormalities.

We demonstrated the occurrence of cephalic hemorrhage in the \textit{itg}α4 mutant zebrafish. Previous studies have reported phenotypes of \textit{itg}α4 loss-of-function using another mutant line or morphant; however, these reports focused on traits at a later stage than 48 hpf, and/or did not describe any hemorrhage phenotypes (Yang et al., 2015; Karpanen et al., 2017; Li et al., 2018). These phenotypic differences may be caused by genetic compensation (El-Brolosy et al., 2019). However, we do not
exclude the possibility that previous investigators were not focused on the cardiovascular phenotype. Nevertheless, this is the first report of hemorrhage as the primary effect of itga4 deletion in zebrafish. We previously revealed a similar hemorrhage phenotype with an itgβ1b mutant and an endothelial cell-specific Itgb1 inhibition transgenic line (Iida et al., 2018). The activity of ITGB1 in endothelial cells is also required for vascular development and preservation of stability, as demonstrated using a mammalian model (Yamamoto et al., 2015). Furthermore, ITGα4β1 expressed in endothelial cells is required for blood vessel maturation in vitro (Garmy-Susini et al., 2005). Therefore, Itga4 is a candidate to partner with Itgb1 to prevent incidental hemorrhage via regulation of blood vessel formation and/or maintenance in zebrafish development.

As described above, embryonic lethality due to placental and cardiovascular abnormalities is observed in Itga4 null mice (Yang et al., 1995; Grazioi et al., 2006). However, there were no apparent defects that could lead to lethality, except cephalic hemorrhage, in the itga4 ko108 homozygous zebrafish embryos. Some mutant zebrafish grew to adulthood and produced offspring. We hypothesized that the gene has undergone functional divergence between zebrafish and mice (Fay and Wu, 2003; Gu, 2003). One possibility is that gain- or loss-of-function occurred in the itga4 of both or either taxa. Another is that zebrafish obtained an alternative paralogous gene(s), via whole-genome duplication, which works as a redundant factor (Imai et al., 2001). However, the itga4 used in this study is currently the only identified gene in zebrafish that is orthologous to the mammalian Integrin α4 gene; thus, we support the former hypothesis, namely an alteration in the gene’s function.

In this study, we generated the itga4 ko108 mutant in zebrafish, and reported the experimental procedures and a phenotypic overview. Our phenotypic analysis suggests a functional divergence of Integrin α4 between zebrafish and mammals. However, as described, the zebrafish is a convenient model to investigate the mechanisms of blood vessel formation and functional diversity in vertebrates.

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