Characterization of biklf/klf17-deficient zebrafish in posterior lateral line neuromast and hatching gland development

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Krüppel-like factors (Klfs) are highly conserved zinc-finger transcription factors that regulate various developmental processes, such as haematopoiesis and cardiovascular development. In zebrafish, transient knockdown analysis of biklf/klf17 using antisense morpholino suggests the involvement of biklf/klf17 in primitive erythropoiesis and hatching gland development; however, the continuous physiological importance of klf17 remains uncharacterized under the genetic ablation of the klf17 gene among vertebrates. We established the klf17-disrupted zebrafish lines using the CRISPR/Cas9 technology and performed phenotypic analysis throughout early embryogenesis. We found that the klf17-deficient embryos exhibited abnormal lateral line neuromast deposition, whereas the production of primitive erythrocytes and haemoglobin production were observed in the klf17-deficient embryos. The expression of lateral line neuromast genes, klf17 and s100t, in the klf17-deficient embryos was detected in posterior lateral line neuromasts abnormally positioned at short intervals. Furthermore, the klf17-deficient embryos failed to hatch and died without hatching around 15 days post-fertilization (dpf), whereas the dechorionated klf17-deficient embryos and wild-type embryos were alive at 15 dpf. The klf17-deficient embryos abolished hatching gland cells and Ctsl1b protein expression, and eliminated the expression of polster and hatching gland marker genes, he1.1, ctsl1b and cd63. Thus, the klf17 gene plays important roles in posterior lateral line neuromast and hatching gland development.
Recent studies demonstrate that genetically gene-disrupted mutants and morphants induced by transient morpholino injection often exhibit distinct morphological phenotypes. In fact, morpholino injection in zebrafish embryos often causes ectopic p53 induction. Furthermore, undesirable off-target effects mediated by morpholino injection have been reported. Transient knockdown analysis using morpholino injection is unsuitable for continuous in vivo analysis; therefore, we have generated the klf17-disrupted zebrafish mutants using the CRISPR/Cas9 technology and examined the continuous physiological function of klf17 throughout early embryogenesis.

Results

Primitive haematopoiesis in the klf17-deficient embryos. To examine the physiological function of klf17 during zebrafish early embryogenesis, we established the klf17-disrupted zebrafish lines using the genome editing technology, CRISPR/Cas9. Three klf17 alleles (uy21, uy22 and uy23) with totally 20 base pairs (bp), 20 bp deletions and a 28 bp insertion, respectively, were isolated (Supplemental Figs S1 and S2). Because the predicted Klf17 mutant proteins derived from mutant alleles lacked most of the coding domains including zinc fingers (Supplemental Fig. S3), they would be functionally disrupted.

Knockdown analysis of the klf17 gene using antisense morpholino suggests that the zebrafish klf17 gene is involved in primitive erythropoiesis development. Therefore, we first examined the production of primitive erythrocytes monitored by gata1:mRFP transgene and haemoglobin production by o-dianisidine staining in the klf17-deficient embryos. The number of erythrocytes on the yolk seemed to be comparable in wild-type (n = 19) and klf17-deficient embryos (mutant) (n = 8) at 25 hpf, whereas primitive erythrocytes were decreased in the klf17-morphant (n = 19) (Fig. 1). Production of haemoglobin and circulating blood cells were detected in the klf17-deficient embryos (n = 10) and in wild-type embryos containing intact alleles (n = 8) at 36 hpf (Figs 1, S4 and Supplemental Movies 1–3). Furthermore, the expression of erythrocyte genes, gata1, /globin (an embryonic globin) at 18-somite and 25 hpf was comparable in wild-type and klf17-deficient embryos (Supplemental Fig. S5). The expression of myeloid cell marker lysozyme C (lyz) was comparably expressed in wild-type and the klf17-deficient embryos at 25 hpf. Thus, we did not observe the severe impairment of primitive erythropoiesis reported in the analysis of the klf17-morphant.

Lateral line neuromast development in the klf17-deficient embryos. Because klf17 gene is bilaterally expressed in the lateral line neuromasts, we examined the differentiation of lateral line neuromasts in the klf17-deficient embryos at 54 hpf using the fluorescent reagent 4-Di-2-ASP (Di-ASP) that can easily label differentiated neuromasts. The posterior lateral line (PLL) primordium migrated caudally and periodically deposited neuromasts at regular five or six intervals and two or three terminal neuromasts (term) along the horizontal myoseptum of wild-type embryos. Deposition of the first PLL neuromast in the klf17-deficient embryos was posteriorly delayed (Fig. 2). The number of PLL neuromasts in the klf17-deficient embryos (n = 9) was less compared to...
that of wild-type (n = 16). The distance between first and second PLL neuromasts was short in the klf17-deficient embryos. Using visualization of alkaline phosphatase accumulation, the delay of the PLL neuromasts deposition was observed in the klf17-deficient embryos (n = 5) compared to wild-type embryos (n = 15) (Fig. 2e,f). Injection of klf17 mRNA (20 pg) in wild-type and the klf17-deficient embryos caused to axis defects at 36 hpf (Supplemental Fig. S6). The expression of lateral line neuromast genes, klf17 and s100t, in the PLL neuromasts was located posteriorly in the klf17-deficient embryos (Fig. 2g–j). Thus, the klf17 gene is required for proper PLL neuromasts deposition.

Hatching gland development in the klf17-deficient embryos. The klf17 gene is detected in the polster during gastrulation stages and in the hatching gland during organogenesis stages. We found that the klf17-deficient embryos (n = 19) failed to hatch at 3 days post-fertilization (dpf) and 6 dpf, whereas wild-type embryos (n = 17) hatched until 3 dpf (Figs 3 and S4). Such hatching defects were obvious and are also observed in the klf17-morphant. The transient klf17-morphant is unsuitable for continuous in vivo analysis. We manually removed the chorion membranes from the klf17-deficient embryos and grew up them. We found that the klf17-deficient embryos (n = 20) died without hatching approximately 15 dpf (Fig. 3). Wild-type embryos (n = 12) and the dechorionated-klf17-deficient embryos (n = 19) were alive. Therefore, we examined the function of klf17 in hatching gland development.

Hatching gland cells in the klf17-deficient embryos. Hatching gland cells in zebrafish are located deep to the enveloping layer on the pericardial membrane. We examined the morphology of hatching gland cells using cross sections of wild-type and the klf17-deficient embryos at 48 hpf. Hatching gland cells visualized by haematoxylin and eosin (HE) staining were observed in wild-type embryos (n = 9), whereas the klf17-deficient embryos (n = 10) were completely missing the hatching gland cells (Fig. 4). Next, we examined the protein expression of Cathepsin L 1b (Ctsl1b) that is one of hatching enzymes. Using anti-Ctsl1b immunohistochemistry, Ctsl1b protein was predominantly expressed in the hatching gland cells of wild-type embryo (Fig. 5). In clear contrast to the wild-type, Ctsl1b expression was not detected in the klf17-deficient embryos.
Next, we examined the expression of polster and hatching gland genes. Consistent with the morphological hatching gland defects, the expression of het1.1 (hatching enzyme 1), ctsl1b, cd63 and klf17 in the klf17-deficient embryos was reduced in the polster of the bud stage embryos (Fig. 6), and was not detected in the hatching gland at 25 hpf (Fig. 7). We examined morphology of polster at bud stage. We found that the polster was not detected in the klf17-deficient embryo (Supplemental Fig. S7). These results suggested that the klf17 gene plays important roles in the polster and hatching gland development in zebrafish.

Discussion

Transient knockdown analysis of klf17 suggests the involvement of klf17 in primitive erythropoiesis and hatching gland development in zebrafish11–13. In this study, we have generated the klf17-deficient zebrafish lines and examined the physiological function of klf17 during early embryogenesis. Although the klf17-morphant exhibits severe defects in primitive erythropoiesis11,13, both primitive erythrocytes and haemoglobin production in the klf17-deficient embryos were observed (Figs 1, S4 and Supplemental Movies 1–3). Reasons of these discrepancies of haematopoietic defect between the klf17-morphants and klf17-deficient embryos are not clear at present. Recent studies found that klf1, klf2a, klf3, klf6a and klf8 in addition to klf17 are expressed in the ICM20. Knockdown of klf3 or klf6a induced a blockage of erythrocyte maturation. One possible explanation is that other klf genes expressed in the ICM function redundantly in primitive erythropoiesis in zebrafish. Multiple klf genes disruption based on the klf17-deficient zebrafish lines will be required to clarify the possibility.

Although klf17 is known to be bilaterally expressed in lateral line neuromasts18, the function of klf17 in lateral line neuromasts is not fully understood. PLL primordium migrates along the horizontal myoseptum and periodically deposits 5 or 6 neuromasts and finally forms 2 or 3 terminal neuromasts at the tip of the tail19. Di-ASP staining analysis revealed that the deposition of first PLL neuromast was delayed posteriorly (Fig. 2). The number of PLL neuromasts was less and the distance between first and second PLL neuromasts was short in the klf17-deficient embryos. The delayed deposition of PLL neuromasts was also confirmed by visualization of alkaline phosphatase accumulation in differentiated PLL neuromasts. Furthermore, the expression of klf17 and s100 at 48 hpf was detected in PLL neuromasts abnormally positioned at short intervals. Because the other klf genes except for klf17 are not detected in lateral line neuromasts, the induction of mutated form of klf17 mRNA in the klf17-deficient embryos may affect the deposition and differentiation of PLL neuromasts. Although the molecular mechanism of PLL neuromast differentiation remains unclear, this study demonstrates that the klf17 gene is required for proper PLL neuromast deposition.

The klf17 is predominantly expressed in the hatching gland in zebrafish and Xenopus2–9. We found that the klf17-deficient embryos failed to hatch during embryogenesis (Figs 3 and S4). Such a hatching defect is consistent with that of zebrafish klf17-morphants and Xenopus klf17-morphants13,14. Our continuous observation revealed that the klf17-deficient embryos died without hatching approximately 15 dpf. Notably, the dechorionated-klf17-deficient embryos were alive at 15 dpf (Fig. 3). The zebrafish klf3 gene is weakly expressed compared with the expression of klf17 in the hatching gland, but the other klf genes except for klf3 and klf17 are not detected in the hatching gland20; therefore, the klf17 would play an essential role in the hatching gland.
Using mammalian cell lines, \( Klf17 \) was recently identified as a negative regulator of metastasis in breast cancer\(^6\). \( Klf17 \) is predominantly expressed in testis and ovary in mice\(^4\); however, the physiological function of mammalian \( Klf17 \) is not fully understood. Because the hatching gland does not exist in mammals, further loss-of-function analysis using \( Klf17 \)-disrupted mice will be required to understand the developmental function of mammalian \( Klf17 \).

Cross section analysis of HE-stained zebrafish wild-type embryos identified the presence of hatching gland cells with cytoplasmic granules\(^22\), presumably containing hatching enzymes that digest the chorion membrane. In clear contrast to that analysis, the hatching gland cells were not observed in the \( klf17 \)-deficient embryos (Fig. 4). Anti-Ctsl1b immunohistochemistry revealed that the Ctsl1b protein, which is one of hatching enzymes, was not detected in the \( klf17 \)-deficient embryos (Fig. 5). Furthermore, the expression of polster and hatching gland marker genes, \( he1 \), \( ctsl1b \) and \( cd63 \) was reduced in the \( klf17 \)-deficient embryos at the bud stage and 25 hpf (Figs 6 and 7). The polster at the bud stage was not detected in the \( klf17 \)-deficient embryo. Therefore, the \( klf17 \) gene is an indispensable transcription factor for the polster and hatching gland development in zebrafish. If the development. Using mammalian cell lines, \( Klf17 \) was recently identified as a negative regulator of metastasis in breast cancer\(^6\). \( Klf17 \) is predominantly expressed in testis and ovary in mice\(^4\); however, the physiological function of mammalian \( Klf17 \) is not fully understood. Because the hatching gland does not exist in mammals, further loss-of-function analysis using \( Klf17 \)-disrupted mice will be required to understand the developmental function of mammalian \( Klf17 \).

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**Figure 4.** Loss of hatching gland cells in the \( klf17 \)-deficient embryos. (a–c) Cross sections of haematoxylin and eosin (HE)-stained embryos at 48 hpf: wild-type \( klf17^{+/+} \) (a) \( klf17 \)-deficient embryos: \( klf17^{uy21uy22} \) (b) and \( klf17^{uy22uy23} \). (c) Hatching gland cells observed in wild-type embryo were missing in the \( klf17 \)-deficient embryos. Arrowheads indicate the position of hatching gland cells. Genomic DNA was isolated from individual caudal fins, with genotyping was performed by genomic PCR. Scale bar, 100 μm.
physiological function of the klf17 gene is conserved among fish, the disruption of fish klf17 genes mediated by genome editing technologies may be useful for eliminating invasive alien fish in closed areas.

**Methods**

**Ethics statement.** All animal experiments were performed in accordance with the animal protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Yamanashi. The IACUC of the University of Yamanashi approved this study (Approval Identification Number: A25–28).

**Synthetic crRNA and tracrRNA, recombinant Cas9 protein and microinjection.** To disrupt the targeted klf17 genomic locus, we used the ready-to-use CRISPR/Cas9 system composed of CRISPR RNA (crRNA), trans-activating crRNA (tracrRNA) and recombinant Cas9 protein. Synthetic crRNAs and tracrRNA (Supplementary Table S1), and recombinant Cas9 protein were obtained from the Integrated Device Technology, Inc (IDT). Synthetic klf17-crRNA1 (25 pg), klf17-crRNA2 (25 pg) and tracrRNA (100 pg) were co-injected together with recombinant Cas9 protein (1 ng) into 1-cell stage zebrafish embryos. Klf17-morpholinos (klf17-MO,
5′-TGCAAATGTTAGGGAATGTCAGAAGG-3′) were injected at one-cell stage embryos as described previously. klf17 mRNA (20 pg) was injected into blastomere at one-cell stage embryos.

**Genotyping for the klf17 locus and genomic sequencing.** To prepare the genomic DNA, the embryos at indicated stages were incubated in 108 μl of 50 mM NaOH at 98 °C for 10 min. Subsequently, 12 μl of 1 M Tris-HCl (pH 8.0) was added to the solution. Genomic fragments at the targeted sites were amplified by PCR with PrimeTaq (GENETBIO Inc.) and the locus-specific primers are listed in Supplementary Table S2. The PCR conditions were as follow: 40 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s. To perform heteroduplex mobility assay (HMA) for genotyping, the resultant PCR amplicons were electrophoresed on a 12.5% polyacrylamide gel. To confirm individual mutations, genomic fragments for the targeted genomic locus were amplified from the solution (1 μl) using PCR (Supplementary Table S2). The resultant PCR fragments were sub-cloned into the pGEM-T Easy vector (Promega) and genomic sequences were determined by sequence analysis.

**Lateral line neuromast labeling.** Lateral line neuromasts at the 54 hpf stage embryos were labeled by incubation of live fish with 0.5 mM 4-(4-diethylaminostyryl)-N-methylpyridinium iodide (4-Di-2-ASP) in E3 (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2 and 0.33 mM MgSO4) medium for 7 min. Labelled fish were washed 3 times with E3 medium and anaesthetized with tricaine (3-amino benzoic acid ethylester), and subsequently were observed under fluorescence microscope. For lateral line neuromast labeling with alkaline phosphatase, the embryos at 54 hpf were fixed in 4% paraformaldehyde for 3 h at room temperature and washed with phosphate buffered saline plus 0.1% Tween-20 (PBST). The embryos were developed in alkaline phosphate buffer containing NBT and BCIP (Nacalai tesque) for 30 min.

**Histological analysis.** Embryos were dehydrated in a graded series of ethanol and embedded using a Technovit 8100 kit (Kulzer). Embedded embryos were sectioned on a Leica RM2125 microtome at 6 μm and mounted on slides. Embryos were stained with haematoxylin-eosin (HE) after sectioning.

**Whole-mount immunohistochemistry.** Embryos were incubated with anti-Cathepsin L 1b (GeneTex, Inc.) at 4°C overnight in PBST containing 5% sheep serum and washed 4 times with PBST. Subsequently, embryos were incubated with Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen) at room temperature for 4 h in PBST containing 5% sheep serum and washed 4 times with PBST.

**Whole-mount in situ hybridization (WISH).** We examined the expression of s100t27, he1.1, ctsl1b28, cd6329, klf17, gata1, β-globin and lyz. Whole-mount in situ hybridization (WISH) was performed as previously described. Zebrafish embryos hybridized with the digoxigenin (DIG)-labelled RNA probe were incubated with alkaline phosphatase-conjugated anti-DIG antibody. To visualize the RNA probe recognized by the anti-DIG antibody, the embryos were subsequently incubated with BM Purple (Roche) as the substrate. Washing the embryos with PBST terminated the colour reaction, and the embryos were fixed in 4% paraformaldehyde.

**Figure 7.** The expression of hatching gland genes in the klf17-deficient embryos at 25 hpf. (a,c,e,g) Wild-type embryos at 25 hpf. (b,d,f,h) klf17-deficient embryos at 25 hpf. Ventral view, anterior up. Arrowheads indicate the position of the hatching gland. After taking pictures, genotyping of individual embryos was performed by genomic PCR. Scale bar, 200 μm.
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Author Contributions

A.K. conceived and designed the work and wrote the manuscript. H.S., T.I., K.Y., R.S., Y.S., R.O. and A.K. performed the experiments. All authors performed the data analysis and reviewed the manuscript.

Additional Information

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