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Aminolevulinate synthase 2 mediates erythrocyte differentiation by regulating larval globin expression during *Xenopus* primary hematopoiesis

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ABSTRACT

Hemoglobin synthesis by erythrocytes continues throughout a vertebrate's lifetime. The mechanism of mammalian heme synthesis has been studied for many years; aminolevulinate synthase 2 (ALAS2), a heme synthetase, is associated with X-linked dominant protoporphyria in humans. Amphibian and mammalian blood cells differ, but little is known about amphibian embryonic hemoglobin synthesis. We investigated the function of the *Xenopus alas2* gene (*Xalas2*) in primitive amphibian erythrocytes and found that it is first expressed in primitive erythroid cells before hemoglobin alpha 3 subunit (*hba3*) during primary hematopoiesis and in the posterior ventral blood islands at the tailbud stage. *Xalas2* is not expressed during secondary hematopoiesis in the dorsal lateral plate. Hemoglobin was barely detectable by o-dianisidine staining and *hba3* transcript levels decreased in *Xalas2*-knockdown embryos. These results suggest that *Xalas2* might be able to synthesize hemoglobin during hematopoiesis and mediate erythrocyte differentiation by regulating *hba3* expression in *Xenopus laevis*.

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1. Introduction

Vertebrate hematopoietic development generally has two phases that are primitive and definitive. In mice, progenitors of the primitive erythrocyte lineage arise from the mesoderm during gastrulation in the yolk sac region, called the blood island [1,2]. Subsequently, the definitive hematopoiesis including the production of primitive erythrocytes occurs first in the aorta-gonad-mesonephros, second in the placenta during embryogenesis, and finally in the fetal liver [3]. Primitive hematopoiesis results in the formation of erythroblasts, megakaryocytes and macrophages in the yolk sac [4]. These erythrocytes have nuclei and hemoglobin consisting of a heterotetramer of two α- and β-subunits of globin containing heme. In this stage, the β-subunit globin is of the fetal type, εγ-globin. Enucleated erythrocytes are produced at the definitive hematopoiesis stage in the fetal liver. After birth, β-subunit hemoglobin of the adult type is produced in enucleated erythrocytes in the bone marrow [3].

Zebrafish, *Xenopus*, and chicken or quail are frequently used as models of hematopoiesis because of species-specific advantages and features. *Xenopus laevis* is particularly useful for large-scale genetic screens and functional analyses. Eggs are available year-round and artificial fertilization is easy. In addition, there is extensive knowledge of the *Xenopus* cleavage patterns and fate map. Moreover, the transparent tadpole is suitable for observing blood cells. The primitive hematopoiesis in *X. laevis* begins at the neurula stage mesoderm in ventral blood islands (VBIs). The VBI is equivalent to the yolk sac blood island of amniotes. It produces a primitive erythroblast and is released and circulated into peripheral blood vessels through the vitelline vein. VBIs have anterior and posterior regions. Progenitors of the anterior VBI (aVBI) are derived from the dorsal marginal zone and progenitors of the posterior VBI (pVBI) are derived from the ventral marginal zone [5]. Myeloid genes such as *spiB* and *mpo* are expressed in aVBI and erythroid genes such as *runx*, *lmo2*, and *scl* (*tal1*) are expressed in pVBI during the neurula stage [6,7]. During embryogenesis, the pVBI expresses the mature erythrocyte marker, larval globin. The dorsolateral plate (DLP) is formed in the early tailbud following gastrulation and is the site of definitive hematopoiesis [8]. The DLP mesoderm is located in the region from which the mesonephros anlage is generated, and produces hematopoietic stem cells.

Abbreviations: ALA, aminolevulinic acid; ALAS2, aminolevulinate synthase 2; DLP, dorsolateral plate; MO, morpholino antisense oligos; VBI, ventral blood island.

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The mechanism of hemoglobin synthesis in erythrocytes has been studied in detail in mammals for many years. The heme synthesis pathway is evolutionarily conserved in vertebrates, and δ-aminolevulinic acid (ALA) is prepared synthetically from succinyl-CoA and glycine in the first reaction of heme synthesis [9]. The enzymes catalyzing this reaction are aminolevulinic acid synthases (ALASs). Two ALAS isozymes are encoded by *ALAS-1* (also known as nonspecific ALAS), which is nonspecifically expressed in adult mammals, and *ALAS-2* (also known as erythroid ALAS), which is specifically expressed in erythroid cells [10]. δ-ALA production is followed by a catalytic series that leads to the formation of heme. The catalytic series is mediated by ALA dehydratase, porphobilinogen deaminase, uroporphyrinogen III synthase, uroporphyrinogen decarboxylase, coproporphyrinogen oxidase, protoporphyrinogen oxidase, and ferrochelatase in the mitochondria and cytosol [9]. Heme regulates several biological processes through heme-responsive or heme sensor proteins [11]. Hemoglobin consists of a heterotetramer of two α- and β-subunits of globin containing synthesized heme in a complex with divalent iron (FeII) and porphyrin. ALAS2 is important enzyme of heme synthesis in erythrocyte and deletion of C-terminal of *ALAS2* causes X-linked dominant protoporphyria in humans [12].

It is unclear whether ALAS have functions other than heme synthesis. Amphibian blood cells differ in some ways from mammalian blood cells and little is known about embryonic heme synthesis in amphibians. We here show that *Xenopus alas2* (*Xalas2*) is first expressed in primitive erythroid cells during primary hematopoiesis before transcription of embryonic globin and is not detected in the DLP during secondary hematopoiesis. This is the first report on hemoglobin metabolism-related gene expression in *X. laevis*.

2. Materials and methods

2.1. Animals

This study was conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan and was approved by the National Institute of Advanced Industrial Science and Technology as part of a project for development of an organ-formation roadmap and application of differentiation technology (No. 0000187-549).

2.2. Embryo culture and manipulation

X. laevis eggs were obtained by injecting adult frogs with 200 μL 100 U/mL Gestoron (Kyoritsu Seiyaku), then artificially fertilized, cultured [13], and staged according to Nieuwkoop and Faber [14]. The embryos were dejellied with 1% sodium thioglycollate [15]. The dejellied embryos were washed several times with 10% Steinberg's solution, then injected with RNAs (1 ng) or morpholino antisense oligonucleotides (MO, 40 ng) in 1 × high-salt modified Barth's saline (MBSH)/5% Ficoll solution in the ventral or dorsal vegetal side of the 8-cell-stage embryo. Embryos were cultured with 10% Steinberg's solution.

2.3. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from embryos and adult organs by phenol methods [15]. First-strand cDNA was synthesized from 1 μg total RNA using an oligo (dT) primer and SuperScript™ III RT (Life Technologies) in a 50 μL reaction. The cDNA (2 μL) was used as a template for RT-PCR. The *Xenopus odc* gene was used as an internal control. Primer sequences are listed in Table 1.

Table 1
Primer sequence for RT-PCR.

Gene name (Fig. 2)	Primer sequence
*Xalas2*a	Fw: 5′-ATCTTCACAACAAGGATGCA-3′ Rv: 5′-GTTATATTGGGAAAGGAGGAC-3′
*Xalas2*b	Fw: 5′-GCCTGAAGAAGAAATTTCTAG-3′ Rv: 5′-AAAGCAGGAGGAGAAAAGAAG-3′
odc	Fw: 5′-GTCAATGATGGAGTGTATGGATC-3′ Fw: 5′-TCCAATCCGCTCTCCTGAGCAC-3′

2.4. MO design and activities

The complete *Xalas2* coding sequence was amplified by RT-PCR, digested with *Bam*HI and *Xho*I, and ligated into the pCS2 plasmid. The plasmid was digested with *Not*I and *in vitro*-capped RNA was synthesized by using mMESSAGE mMACHINE® SP6 Kit (Life Technologies). We identified the *Xalas2* 5′ untranslated region (UTR) sequence by 5′ rapid amplification of cDNA ends (RACE; SMARTer™ RACE cDNA Amplification Kit, Clontech) and *Xalas2*-MO was designed in Gene Tools, LLC (https://store.gene-tools.com). MO sequences were as follows: *Xalas2a*-MO, 5′-ACGATTAATGAGAGAAGCCATGTTC-3′ and *Xalas2b*-MO 5′-CAACGATTGATGAGAGAAGCCATGT-3′. We amplified the *Xalas2* coding sequence with the 5′UTR or five point mutations in the morpholino-binding site by RT-PCR with high-fidelity DNA polymerase (*Pfu*, Promega) and the primers listed in Table 2.

We synthesized two types of *Xalas2* mRNA from the pCS2-5′UTR-*Xalas2*-5myc and pCS2-5mis-*alas2*-5myc constructs. To verify the activities and specificities of the MO, we injected these mRNAs and MOs into 2-cell-stage embryos, and then cultured the embryos to stage 9 before freezing. Proteins were extracted from the embryos and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes with an iBlot (Life Technologies) dry blotting system, and incubated with antibodies for SC-40 HRP, Myc 19E10, mouse monoclonal IgG (Santa Cruz Biotechnology), AC-40 (Sigma Aldrich), and the positive control mouse IgG (H&L) HRP-liked antibody (Cell Signaling); antibodies were detected with enhanced chemiluminescence (SuperSignal West Femto, Pierce), and the protein bands were visualized using an LAS1500 Analyzer (FujiFilm).

2.5. RNA probe constructs and whole-mount in situ hybridization

The *Xalas2* complete mRNA sequence clone (GenBank Accession Number BC080015) was purchased as pCMV-SPORT6 *Xalas2* from Thermo Fisher Scientific (Open Biosystems). pCMV-SPORT6 *Xalas2*, pGEM T easy hba3 (NM_001086328), pBluescript II *Gata1* (NM_001085640), and pBluescript II SCL [16] were transcribed with T3 or T7 RNA polymerase and DIG RNA Labeling Mix (Roche). The embryos were processed for whole-mount *in situ* hybridization [17]; some were embedded in paraffin, sectioned at 10 μm, and stained with eosin.

Table 2
Primer sequence for MO design and activities.

Name (Fig. S1)	Primer sequence
Injected *Xalas2*a	Fw: 5′-CCGGATCCAGTGCAGGGCAACAGAAAC-3′ Rv: 5′-CCATCGATCAGAGGCATACATAGTAATGTATTTT-3′
Injected *Xalas2*a 5mis[a]	Fw: 5′-CCGGATCCATGGCATCACTGATAAATCGATGTCCC-3′ Rv: 5′-CCATCGATCAGAGGCATACATAGTAATGTATTTT-3′
Injected *Xalas2*b	Fw: 5′-CTTGACGTGTGAACATGGCTTCTCTCATCAATCG-3′ Rv: 5′-GTGTCTGTTGCCCTGCACTG-3′
Injected *Xalas2*b 5mis[a]	Fw: 5′-CCGGATCCATGGCATCACTGATAAATCGATGTCCC-3′ Rv: 5′-CCATCGATCAGAGGCATACATAGTAATGTATTTT-3′

[a] *Xalas2*a, b 5mis introduces five mutations into *Xalas2a* mRNA.

2.6. Hemoglobin staining of embryos

Whole-embryo staining for hemoglobin expression was performed using o-dianisidine (Sigma Aldrich) histochemistry according to previously described methods [18]. After staining, the embryos were fixed in MOPS/EGTA/magnesium sulfate/formaldehyde buffer (MEMFA) for 3 h and incubated in benzylbenzoate/benzyl alcohol (BB/BA).

3. Results and discussion

3.1. Isolation of Xalas2

Using 5′ RACE, we identified two *Xalas2* coding sequences (*Xalas2a* and *Xalas2b*) and compared their amino acid sequences with those of other vertebrates. It was clear that *Xalas2* contains heme regulatory motifs (CP motif) comprised of five residues, [Arg, Lys or Asn]-Cys-Pro-[Lys or hydrophobic residue]-[Lue or Met] (Fig. 1). Heme binds to the CP motifs of ALAS-1 pre-proteins and of ALAS-2 in the mitochondria [19]. The processing site and CP motif are widely conserved in vertebrates, and mitochondrial transport of preALAS-2 is inhibited by exogenous hemin [20]. We suggest that *Xalas2* shares the same functions and regulatory system as other vertebrates and that its activity may be regulated by heme.

3.2. Xalas2 was expressed in hemangioblasts and erythrocytes

Xalas2a mRNA expression increased from stage 15 and was maintained until stage 42; in contrast, *Xalas2b* expression began at stage 34 (Fig. 2A). *Xalas2* expression was analyzed by *in situ* hybridization to characterize its relationship with erythropoiesis. *Xalas2* expression started in the anterior ventral mesoderm at the neurula stage (Fig. 2B, C), similar to *scl* (Fig. 2K), which encodes a transcription factor that regulates hematopoietic factors [21] and acts in the early ventral mesoderm to specify hematopoietic stem cells in *X. laevis* [22]. *Xalas2* and *scl* expression patterns presented largely the same features such as two crossing lines and pVBI in stage 23 (Fig. 2D, D′ and L). The *hba3* gene is a globin gene for alpha hemoglobin and an erythrocyte marker in embryonic hematopoiesis. At stage 38, the point of vasculogenesis and blood cell circulation, *Xalas2* expression was observed in the aortic arch near the heart and posterior condylar vein (Fig. 2F and F′) narrower than *hba3* (Fig. 2J); *Scl* expression decreased (Fig. 2N). These results suggest *Xalas2* is expressed in circulating erythrocytes (Fig 2F″, J″). Other heme synthetases are present as maternally derived mRNA [23], indicating that *Xalas2* could be a rate-limiting enzyme in *Xenopus*, similar to its function in mammals.

3.3. Inhibition of alas2 translation also inhibited embryonic hemoglobin synthesis

To demonstrate the role of *Xalas2*, we inhibited the translation of the two *alas2* types. The designed *Xalas2*a-MO and *Xalas2b*-MO inhibited translation (Fig. S1). Hemoglobin staining with o-dianisidine confirmed *X*Alas2 function as a heme synthase in the *Xenopus* embryo. Hemoglobin was nearly undetectable in embryos injected with *Xalas2* MOs (Fig. 3B). Succinylacetone is structurally similar to aminolevulinic acid; therefore, it competes with aminolevulinic acid and prevents synthesis of porphobilinogen. The amount of hemoglobin was inversely related to succinylacetone dose (Fig. 3C, D); addition of 0.25 mM succinylacetone abolished hemoglobin synthesis (Fig. 3C). Thus, *Xalas2* MO and succinylacetone prevent hemoglobin synthesis in erythrocytes during tadpole

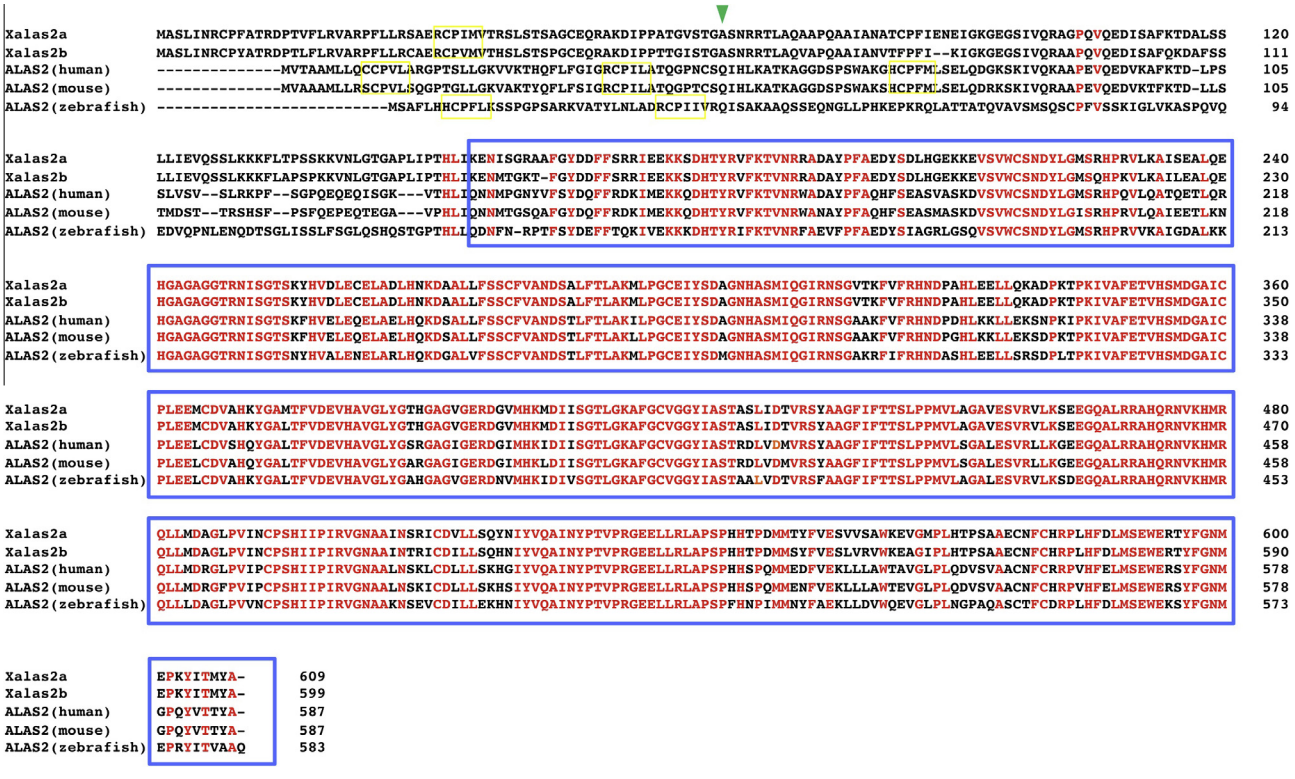

Fig. 1. ALAS2 sequence alignment and identities. Alignment of Alas2 protein sequences from *Xenopus* (type A Accession No. NP_001087499; type B Accession No. AAH84616), human (Accession No. NP_000023), mouse (Accession No. NP_033783), and zebrafish (Accession No. NP_571757). The yellow box indicates the heme regulatory motif (CP motif [Arg, Lys or Asn]-Cys-Pro-[Lys or hydrophobic residue]-[Lue or Met]). The blue box indicates the catalytic core domain. The green arrowhead indicates the site of precursor protein processing by mitochondrial peptidase.

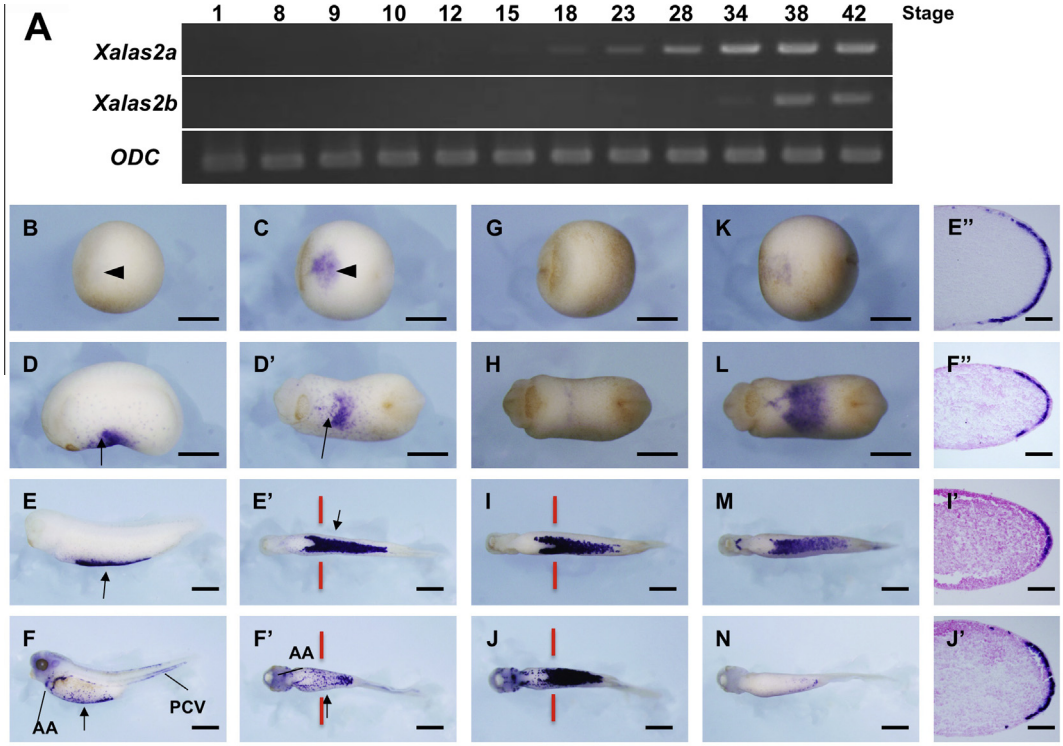

Fig. 2. *Xalas2* and erythrocyte-related gene expression in *X. laevis* embryos. (A) RT-PCR analysis of *Xalas2* type a and b expression during *Xenopus* development. Ornithine decarboxylase (*odc*) served as an internal control. Whole-mount *in situ* hybridization analysis using *Xalas2* antisense probe (B–F, D′–F′, E″, F″), *hba3* (G–J, I′, J′), and *scl* (K–N). (B) Stage 15: ventral view shows weak *Xalas2* expression in the primitive hemangioblast (arrowhead). (C) Stage 18: expression in the primitive hemangioblast is extensive as viewed from the ventral side. (D, D′) Stage 23, (E, E′) stage 34: ventral, lateral views of the same embryo show extensive staining in the ventral blood island (VBI; arrow mark). (F, F′) Stage 38: expression of *alas2* is extensive in the circulatory system (ventral and lateral views). Eosin-stained sections showing *Xalas2* expression in stage 34 (E″) and stage 38 (F″), and *hba3* expression in the same stage (I′, J′). Transverse sections in panels E″, F″, I′, J′ of embryos are from the respective positions of the red lines in panels E′, F′, I, J. DLP, dorsal lateral plate; AA, aortic arch; PCV, posterior cardinal vein. Scale bars in stages 15, 18, and 23 indicate 0.5 mm; in stages 34 and 38, the bar represents 0.1 mm.

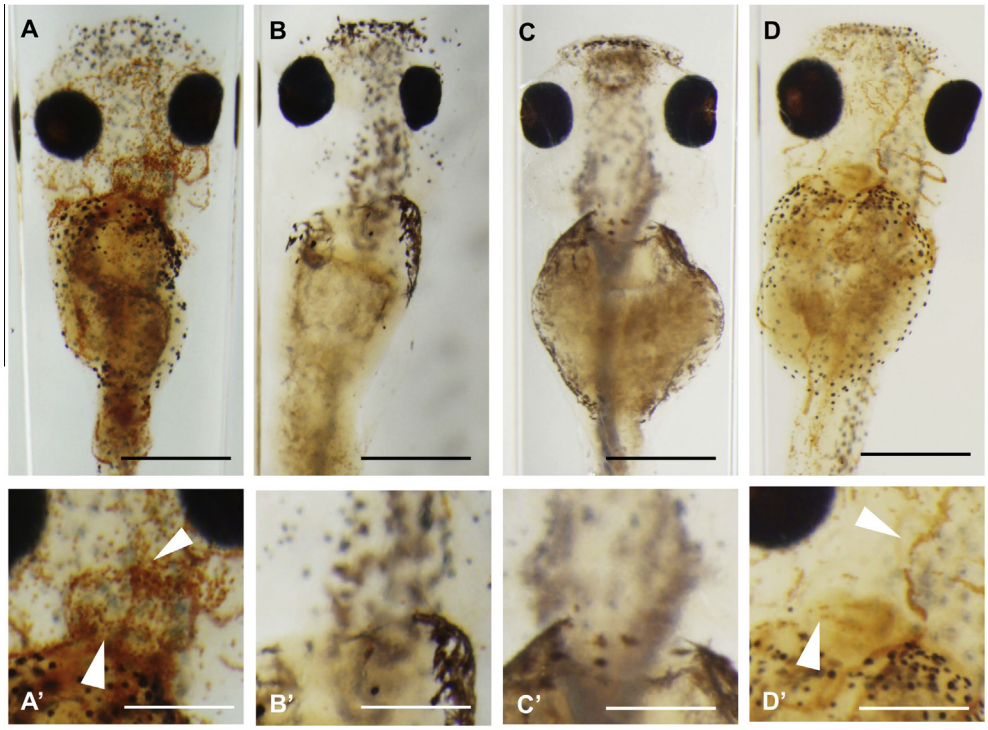

Fig. 3. Hemoglobin staining in *Xalas2*-MO injected and succinylacetone-treated embryos. The control-MO injected embryo (ventral vegetal) (A, A′). Hemoglobin staining in *Xalas2* MO-injected embryo (B, B′), succinylacetone (C, C′, D, D′). Embryo treated with 0.25 mM succinylacetone (C, C′) and untreated embryo (D, D′) from the 8-cell-stage to stage 42. Scale bars in (A–D) and (A′–D′) indicate 0.5 mm and 0.25 mm, respectively. Arrowheads indicate an erythrocyte stained with o-dianisidine.

embryogenesis, demonstrating the conservation of function in mammals.

3.4. Xalas2 regulated hba3 mRNA expression in VBI

The function of *Xalas2* before erythrocyte generation has not been reported elsewhere. When we injected both 8-cell blastomeres in the dorsal-vegetal with *Xalas2a*-MO and *Xalas2b*-MO, an effect was observed for aVBI. In contrast, when the injection was in the ventral-vegetal, the MOs affected pVBI. The *scl* expression in aVBI in embryos injected with *Xalas2* MOs into the dorsal or ventral regions did not differ from that of control MO-injected embryos (Fig. 4A–E). *Scl* expression was hardly detected in the aortic arch in *Xalas2* MO-injected embryos (dorsal-vegetal injection) at stage 34 (Fig. S2). The V-shape expression of *hba3* disappeared at stage 34 in pVBI in embryos that were injected with *Xalas2* MO at the dorsal-vegetal side (Fig. 4G). There was a slight difference in the disrupted expression between control MO and *Xalas2* MO-injected embryos (Fig. 4F–H, Table S1). Upon injection in the ventral vegetal side, the expression of *hba3* in pVBI disappeared more frequently (62–82%) than in the control MO-injected embryo (Fig. 4I–K, Table S2). In the mouse, fetal εy-globin expression is significantly reduced in E10.5 *Alas-2* mutant embryos compared to wild-type embryos [24]. Although Hba3 is an α-globin type, our results suggest that XAlas2 also inhibits expression of *hba3* in pVBI. Thus, inhibition of *Xalas2* translation did not affect primitive erythrocyte marker expression at the neurula stage (Fig. 4A–E), but hematopoietic differentiation and *hba3* expression were inhibited at the tailbud stage (Fig. 4F–K). In *Xenopus* primary hematogenesis, XAlas2 influences embryonic globin gene expression. Gata1 is a hematopoiesis-related transcription factor. The expression of *gata1* at stage 34 was not affected by *Xalas2*-MO injection (Fig. 4L–N), but

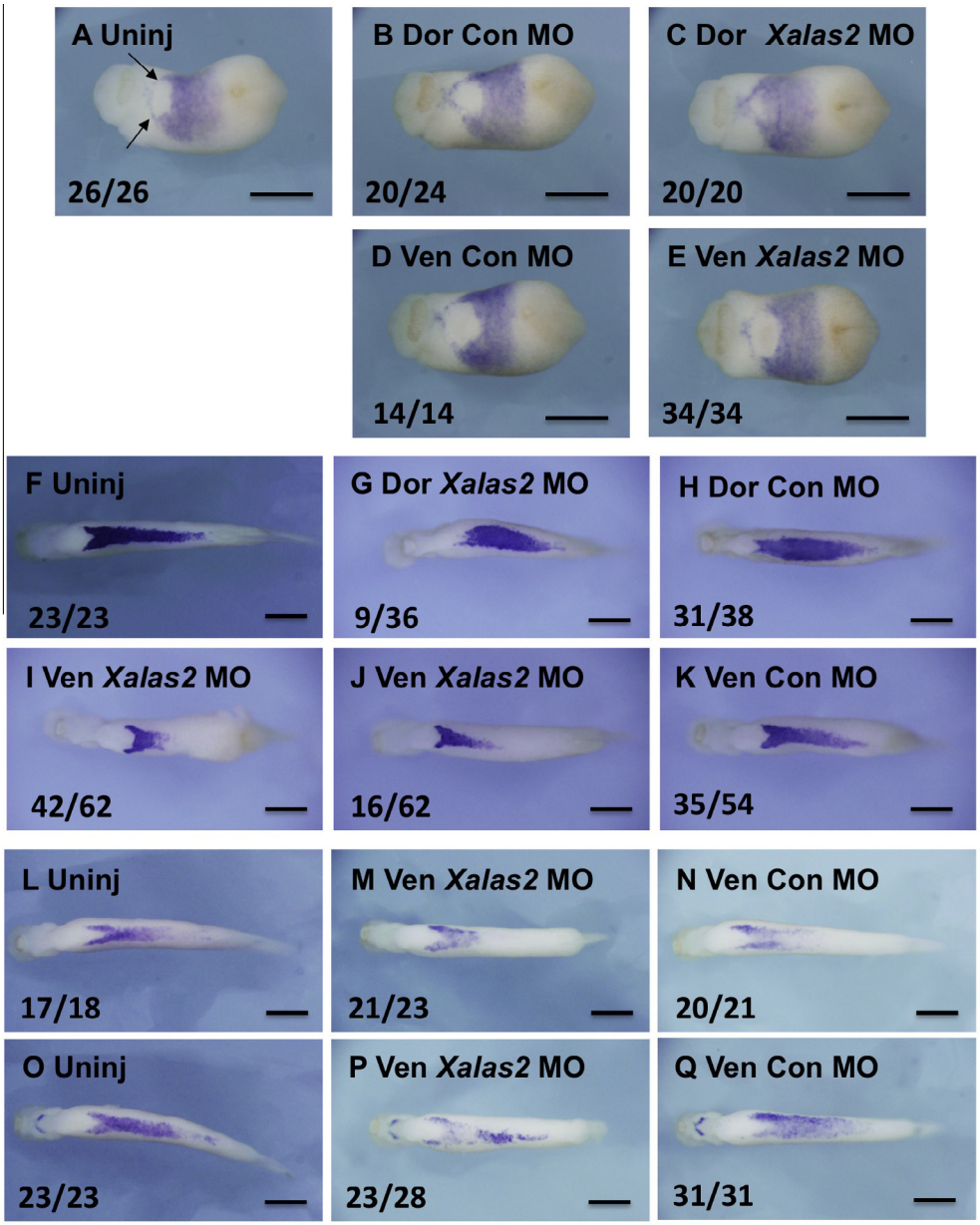

Fig. 4. Erythrocyte gene expression in *Xalas2*-MO injected embryos. Embryos were microinjected in the dorsal-vegetal side (Dor) and ventral-vegetal side (Ven) with 40 ng *Xalas2*-MO. Whole-mount *in situ* hybridization of *scl* in stage 23 (A–E), and *hba3* (F–K), *Gata1* (L–N), and *scl* (O–Q) in stage 34. The arrow indicates two crossing lines (A). The number of phenotypes, which a photograph present per total number of injected embryos is indicated at the lower, left of the each figure. The experiments were performed independently at least two times. Scale bars represent 0.5 mm.

scl expression was downregulated in a portion of the VBI (Fig. 4O–Q). Thus, XAlas2 may not regulate *gata1*, but it does maintain *scl* expression throughout the VBI. XAlas2 or the heme synthesized by XAlas2 may not regulate *hba3* through *gata1* and *scl*; *hba3* expression may be regulated by another pathway [25,26]. This is the first report on heme synthesis and function in *Xenopus* and the mechanism by which *alas2* is associated with the embryonic globin gene remains unclear. The evolution of hematogenesis in animals will be clarified by solving the complicated mechanism of primary hematogenesis in amphibians.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.110.

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