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The zebrafish erythropoietin: Functional identification and biochemical characterization

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Abstract In the present study, the zebrafish *epo* cDNA was cloned. The encoded protein displays 90%, 55% and 32% identity to the Epo from carp, fugu and human, respectively. Through RT-PCR, the expression of *zepo* mRNA was mainly in the heart and liver. In the COS-1 cell transfection experiments, the recombinant zEpo-HA protein was efficiently secreted into the culture medium as a glycoprotein and the carbohydrate moiety can be cleaved by the treatment of peptide-*N*-glycosidase F (PNGase F). Using the morpholino approach, we showed that *zepo* morphants displayed severe anemia leading to high mortality during development. Such an effect can be significantly rescued by *zepo* RNA. Furthermore, in the absence of functional zEpo, the expression of specific markers for adult globin genes, such as α*A1*- and β*A1-globin*, but not the embryonic β*e1-globin*, was affected.

Keywords: Erythropoietin; Zebrafish; Morpholino oligonucleotide; Glycoprotein; Secretion; Globin staining

1. Introduction

Erythropoietin (Epo) is a glycoprotein hormone with the function as a principal regulator of erythropoiesis. In mammals, the initial production organ is liver and is shifted to the kidney after birth [1]. Effects of Epo are mediated by binding to Epo receptor (Epor), which is primarily expressed in hematopoietic progenitor cells [2]. Upon binding of Epo to its receptor, dimerization of Epor leads to the activation of the JAK/STAT signaling pathway [3]. The main function of Epo is to inhibit the apoptosis of erythroid precursor cells and to increase their survival. Currently, recombinant human Epo (rhEpo) has been used to treat anemia caused by chronic kidney disease [4]. Moreover, rhEpo is also used to treat cancer patients with anemia induced by chemotherapy or radiotherapy [5].

The first *erythropoietin* gene from non-mammalian vertebrate has been cloned from fugu and the encoded protein shows only 32% identity to human Epo [6]. Human Epo is a glycoprotein and contains three N-linked sugar chains on Asn24, Asn38 and Asn83 as well as one O-linked sugar on Ser126 [7]. The fugu Epo has no N-linked glycosylation site, but it is postulated to have an O-linked glycosylation site on Ser117 [6]. Site-directed mutagenesis analysis has indicated that the rhEpo variant with either a double mutation (Gln38,83) or a triple mutation (Gln24,38,83) on the three N-glycosylation sites by replacing the asparagines (Asn) with glutamines (Gln) was secreted poorly from COS-1 and CHO cells [8]. The secretion ability of the fugu Epo in cultured cells has not been determined yet.

In this study, we isolated a gene encoding zebrafish Epo (zEpo) that shows 55% and 32% identity to the Epo from fugu and human, respectively. Unlike the fugu Epo, zEpo contains two N-glycosylation sites and it was efficiently secreted into the culture medium as a glycoprotein. Through RT-PCR, the expression of *zepo* mRNA was mainly in the heart and liver. Using morpholino approach, we showed that zEpo morphants displayed severe anemia leading to high mortality during development. Furthermore, in the absence of functional zEpo, the expression of erythroid specific markers, such as adult *globin* genes and the embryonic β*e1-globin* gene were examined.

2. Materials and methods

2.1. Fish

Zebrafish were raised and maintained under standard conditions. Embryos were incubated at 28 °C and the different developmental stages were determined according to the descriptions in the Zebrafish Book [9].

2.2. Total RNA isolation and RT-PCR analysis of zebrafish erythropoietin mRNA

Total RNA was isolated from the fertilized eggs at different stages and various tissues of zebrafish (*Danio rerio*), using the RNAzol reagent (Tel-Test, Friendswood, TX, USA) according to the instructions of the manufacturer. After treatment with RQ1 RNase-Free DNaseI (Promega), 50–100 μg of total RNA was subjected to the first strand cDNA synthesis. PCR amplifications were performed with *zepo* primers (zEPO-RT-F, 5′-GTG CCT CTC ACT GAG TTC TTG GAA G-3′ and zEPO-RT-R, 5′-CTC GTT CAG CAT GTG TAA GCC TGA C-3′) or β-actin primers (zAct-F, 5′-CCT CCG GTC GTA CCA CTG GTA T-3′ and zAct-R, 5′-CAA CGG AAG GTC TCA TTG CCG ATC GTG-3′) and the cDNA as a template.

2.3. Cloning of the full-length cDNAs encoding zEpo and hEpo

The full-length cDNA encoding zEpo was isolated by PCR amplification using gene-specific primers (zEPO-F, 5′-ATG TTT CAC GGT TCA GGA CTC-3′; zEPO-R, 5′-GCT GAC ACC CTG TCG ACA

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GAC-3′) according to the sequence with GenBank accession number EF426727. The full-length cDNA encoding hEpo was isolated by PCR amplification using gene-specific primers (hEPO-F, 5′-ATG GGG GTG CAC GAA TGT CCT GCC-3′; hEPO-R, 5′-TCT GTC CCC TGT CCT GCA GGC CTC-3′) according to the sequence with GenBank accession number NM000799 from human kidney tissue cDNA. The cDNA encoding zEpo or hEpo was subcloned into the BamHI and EcoRI site of pHA-YUN vector [10] to generate pCMV-zEpo-HA or pCMV-hEpo-HA, respectively. The plasmid pCMV-GFP-HA [10] was constructed by inserting the green fluorescence protein (GFP) coding region into pHA-YUN.

2.4. Cell cultures

Monkey kidney fibroblast COS-1 cells were cultured in high-glucose Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (HyClone, UT, USA) in a humidified atmosphere of 5% CO_2 at 37 °C. Cell transfection was carried out using Lipofectamine Plus reagent (Life Technologies) according to the manufacturer's protocol. After transfection for 12 h, cells were incubated in serum-free medium for an additional 36 h. Conditioned media were then collected and concentrated with YM-10 MW Centricon (Millipore, Bedford, MA). The concentrated supernatant was digested with 1 U of peptide *N*-glycosidase F (PNGase F) (Roche) at 37 °C for 18 h. Untreated and *N*-glycosidase-treated culture supernatants were analyzed by SDS–PAGE followed by detection with Western blotting.

2.5. Western blot and immunocytochemistry assay

Cells were harvested at 48 h post-transfection for Western blot and at 24 h post-transfection for immunocytochemistry analysis. Western blot analyses were performed with anti-HA monoclonal antibody (1:3000; Santa Cruz, CA) at 4 °C overnight. The signals were detected using enhanced chemiluminescence (ECL) (NEN Life Science Products, MA). For immunocytochemistry analysis, cells were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. Immunostaining was performed using anti-HA antibody (1:1000) at 4 °C overnight, followed by Cy3-conjugated goat anti-mouse antibody and co-stained with DAPI for 30 min at room temperature. Photoimages were prepared by using a laser scanning confocal microscope (LSM 510 Meta, Carl Zeiss Inc., Germany).

2.6. Morpholino injection

Antisense morpholinos (MOs) were synthesized by Gene Tools (Philomath, OR, USA). The sequence of *zepo*-MO was as follows: 5′-TGA AAC ATT CGC AAA ACA ACT TGG C-3′. The MO was dissolved in 1 × Danieau solution containing 0.5% phenol red to 0.3 mM and 3.2 ng per embryo was injected into embryos at the 1–2 cell stage. The embryos were processed for whole-mount in situ hybridization at 24 hpf or *o*-dianisidine staining at 48 hpf.

2.7. Morpholino rescue experiment by mRNA injection

Capped *zepo* sense RNA was synthesized with the mMESSAGE mMACHINE T7 Ultra kit (Ambion, Austin, USA) according to the manufacturer's protocol from linearized pT7TS plasmids containing the entire coding region of *zepo* cDNA. Ten picograms to 30 pg of sense *zepo* RNA was co-injected with 3.2 ng zEpo-MO per embryo at 1–2 cell stage.

2.8. o-Dianisidine staining

MO-injected or uninjected embryos at 48 hpf were dechorionated and fixed with 4% paraformaldehyde overnight. Fixed embryos were washed in PBS for three times and then incubated in the staining buffer (0.6 mg/mL *o*-dianisidine, 10 mM sodium acetate (pH 5.2), 0.65% hydrogen peroxide, and 40% ethanol) for 15 min in the dark.

2.9. Whole-mount in situ hybridization

Digoxigenin-labeled antisense RNA probes were generated by in vitro transcription using linearized plasmids as template. Whole-mount in situ hybridization were performed as previously described [10]. Specific primers for *epo* (5′-GTG CCT CTC ACT GAG TTC TTG GAA G-3′ and 5′-CTC GTT CAG CAT GTG TAA GCC TGA C-3′), *gata1* (5′-ACC TGA GGC TCG TGA ATG TG-3′ and 5′-GCT CAT CTG GAG GTG CCA TGT-3′), *scl* (5′-TCC TAG CAA TCG AGT CAA GCG-3′ and 5′-TGG ACT CCA CTG ATG AGT CCT G-3′), β*e1-globin* (5′-ACA TGG TTG TGT GGA CAG ACT TCG AG-3′ and 5′-TTA GTG GTA CTG TCT TCC CAG AGC GG-3′), α*A1-globin* (5′-ACG CAG CGA TGA GTC TCT CTG ATA C-3′ and 5′-GCA CAG TGT TGT TGT CAG TGA ATA T-3′) and β*A1-globin* (5′-ACA TGG TTG AGT GGA CAG ATG CCG-3′ and 5′-CAT TGG CGA TGA GAC TCT AGT GGT-3′) were used to amplify the DNA as template.

3. Results

3.1. Cloning of the erythropoietin gene from zebrafish

To identify zebrafish cDNA related to Fugu *epo*, we used the coding region of the Fugu *epo* (GenBank accession number AY303753) to search the GenBank for related expression sequence tag (EST) sequences by using the program tBLAST. Two zebrafish EST clones (GenBank accession numbers DN903417 and EB886593) were found and assembled to obtain the full-length cDNA. Their sequences have been deposited in GenBank with the accession number of EF426727. To determine the genomic structure of zebrafish *erythropoietin* (*zepo*) gene, we used this cDNA as a bait to perform an online BLAST search of the GenBank database and matched five non-contiguous regions in the 200 556 bp zebrafish BAC clone DKEY-46E6. Subsequently, we compared the sequences between this BAC clone and the *zepo* cDNA. The result indicates that the *epo* cDNA is contained within five putative exons and four introns spanning approximately 13.4 kb.

The deduced protein sequences were aligned with other fish Erythropoietins and mammalian Erythropoietins. The zEpo shows an overall identity of 90%, 55%, 32% and 35% to Epo from carp, Fugu, human and mouse, respectively (Fig. 1B). In a previous report, Fugu Epo also shows 32% identity to human Epo [6]. The signal peptide of human Epo has been assigned [7] and the corresponding regions of zEpo were also predicted (Fig. 1B). There are four cysteine residues in mature human Epo that form two disulphide bridges important for the stability and function of the mature protein [11]. These cysteine residues are conserved in all fish Erythropoietins (Fig. 1B). Moreover, human Epo is a glycoprotein and contains three N-linked sugar chains on Asn24, Asn38 and Asn83 [7]. Similarly, the zEpo contains two N-linked glycosylation sites, ^{38}NVT and ^{81}NQT.

3.2. Expression profiles of zepo transcript in different developmental stages and adult tissues in zebrafish

To investigate the expression pattern of *zepo* transcript, zebrafish embryos at different developmental stages and various tissues from adult zebrafish were collected for cDNA preparation and subjected to RT-PCR. In adult zebrafish, the *epo* mRNA is predominantly expressed in the heart and liver, while low expression is observed in the brain, gill, eye, intestine and kidney (Fig. 2A). On the contrary, the kidney is the primary organ to synthesize Epo in adult mammals [1]. This expression pattern is consistent with that of fugu *epo* mRNA, which is predominantly expressed in heart and moderate in liver and brain [6]. During development, the *zepo* transcript was detected in all stages (Fig. 2B).

We also examined the temporal and spatial patterns of *zepo* mRNA expression using whole-mount in situ hybridization. In 24, 48, 72, 96 and 120 hpf embryos (Fig. 2C), *zepo* mRNA was expressed in forebrain, midbrain, hindbrain and brachial arch.

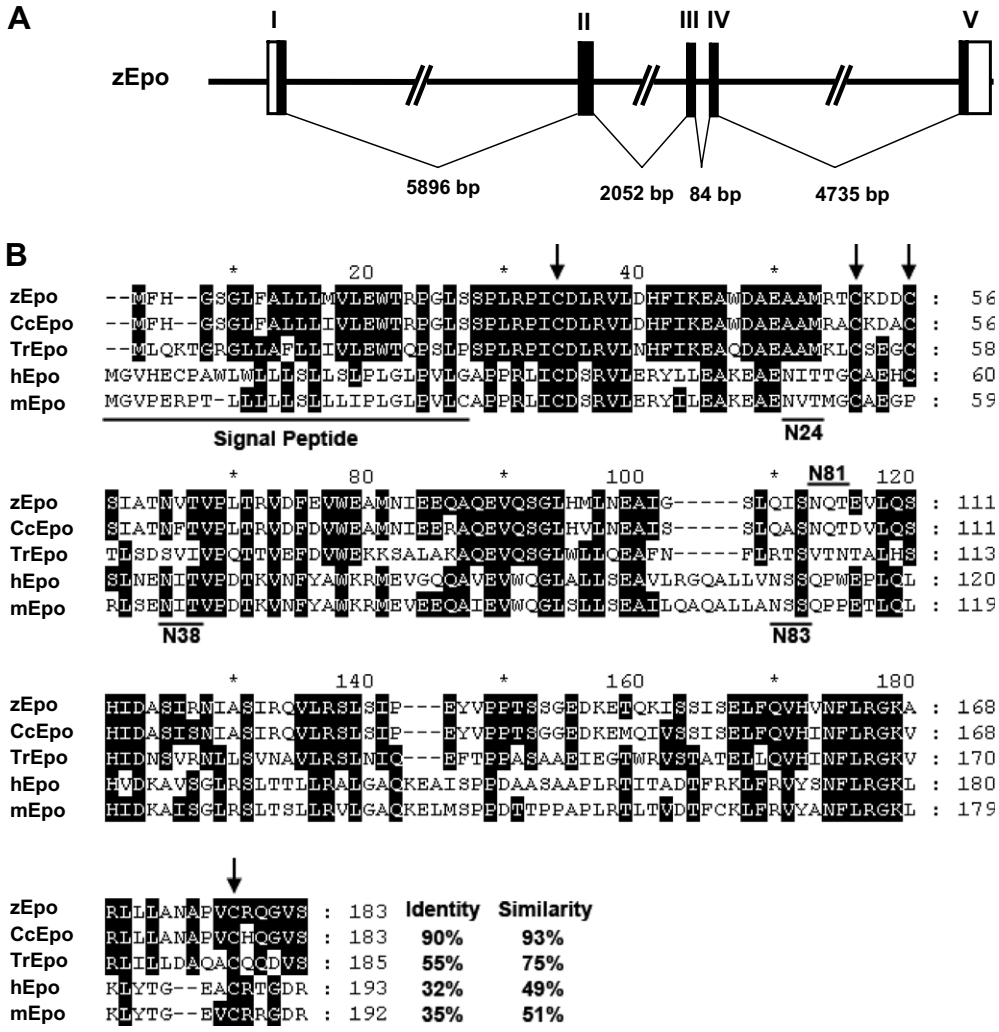

Fig. 1. Genomic organization of the zebrafish *erythropoietin* gene and alignment of amino acid sequences of zEpo with Epos from other species. (A) Genomic structure of the *zepo* gene. Exons are indicated by the boxes numbered from 1 to 5, including the coding regions (solid boxes) and the untranslated regions (open boxes). Introns and the 5′-flanking regions are indicated by the solid lines. (B) Multiple alignment of Epos from fish and mammals using CLUSTAL X: The amino acid sequence of the zebrafish Epo (zEpo; EF426727) was compared with those from carp (ccEpo; DQ278877), Fugu (trEpo; AY303753), human (hEpo; NM_000799) and mouse (mEpo; NM_007942). Identical residues in three proteins are highlighted. Signal peptide and putative N-linked glycosylation sites are underlined or overlined. The conserved four cysteine residues are indicated by arrows.

In addition, *zepo* transcript was detected in early embryos at 12 hpf.

3.3. Secretion of zebrafish Epo in COS-1 cells

Epo is a secreted glycoprotein and the signal peptide of human Epo has been assigned [7]. The putative signal peptide of zEpo contains 23 amino acid residues and those of human and mouse Epo have 27 amino acid residues. Interestingly, eight amino acids of the N-terminal 12 amino acids, APPRLICDSRVL, of mature human Epo are highly conserved between fish and mammalian Erythropoietins.

In order to confirm the secretion ability of zEpo, a HA-tag was added to the C-terminal end of zEpo. At 48 h post-transfection of pCMV-zEpo-HA into COS-1 cells, the fusion protein in cell lysate or conditioned medium was detected by SDS–PAGE and Western blot analysis using a monoclonal antibody against HA-tag. As shown in Fig. 3A, the zEpo-HA was present in both conditioned medium and cell lysate. The molecular mass of secreted zEpo-HA was reduced when it was treated with PNGase F, suggesting that this fusion protein undergoes post-translational modification of glycosylation. As a positive control, the HA-tagged human Epo was also expressed in both conditioned medium and cell lysate and was glycosylated in COS-1 cells.

Due to the properties of secretion and glycosylation, zEpo-HA and hEpo-HA are expected to be localized in the ER/Golgi. Immunofluorescence studies of COS-1 cells transfected with pCMV-zEpo-HA using the anti-HA monoclonal antibody revealed that zEpo-HA was localized in the perinuclear region and displayed a punctate, non-uniform expression pattern, suggesting that it is localized to ER/Golgi (Fig. 3B). Similarly, hEpo-HA was localized to ER/Golgi of COS-1 cells as well. As a control, GFP-HA was localized to the cytosol with a uniform distribution.

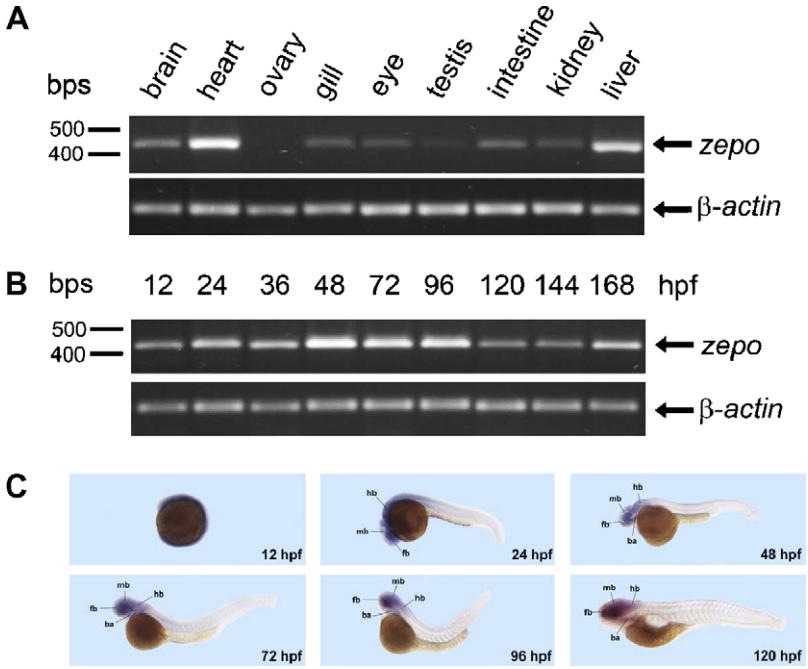

Fig. 2. Expression profiles of *zepo* mRNA by RT-PCR and by whole-mount in situ hybridization. RT-PCR of *zepo* transcript was performed with a pair of primers producing a DNA fragment of 438 bp. β-actin bands were used to normalize the amount of cDNA prepared from different tissues (A) and from embryos at different developmental stages (B). Whole-mount in situ hybridization with antisense *zepo* at different developmental stages was performed (C). The images were taken from a lateral view, with anterior to the left and dorsal to the top. fb, forebrain; mb, midbrain; hb, hindbrain; ba, brachial arch.

3.4. *Knockdown of zepo resulted in strong suppression of hemoglobin production in zebrafish embryos and reduction of erythroid-specific gene expression*

Morpholino (MO)-mediated knockdown of genes in zebrafish embryos has become a routine and efficient method to provide information about gene function in vivo [12]. To examine the function of zEpo in the regulation of hemoglobin production, we injected *zepo*-MO into zebrafish embryos at 1–2 cells stage. In Fig. 4A, the presence of hemoglobin in zebrafish embryos was detected by o-dianisidine staining [13]. Embryos injected with *zepo*-MO at 48 hpf displayed significant loss of hemoglobin (panels b and b′), as compared to that of wild-type embryos (panels a and a′). Moreover, the mortality rate of *zepo*-MO-injected embryos was very high (80% mortality, $n = 250$), possibly due to the significant loss of hemoglobin and other Epo-responsive effect during development [14]. In addition, obvious pericardial edema was observed in the EPO-MO-injected embryos. To test whether *zepo* RNA can rescue this defect, we co-injected *zepo*-MO and *zepo* RNA into zebrafish embryos at the one-cell stage. Our results showed that the mortality rate was reduced to approximately 30% ($n = 150$) and *zepo* RNA-injected embryos displayed obvious hemoglobins stained by *o*-dianisidine (panels c and c′).

To further examine the role of Epo during primitive erythrogenesis, we analyzed the effect of *zepo*-MO injection on the expression of erythroid-specific genes, such as α*A1-globin*, β*A1-globin*, βe1-*globin*, *scl* and *gata1* by whole-mount in situ hybridization. There are five adult globin genes in zebrafish, three α-*globin*, and two β-*globin* genes [15], while only one embryonic globin gene has been identified, a β-*like globin* gene (βe1) [16]. In addition, the stem cell marker *scl* [17] and the immature erythroblast marker *gata1* [13] were also examined. As shown in Fig. 4B, there was no difference in the expression of *scl*, *gata1* and β*e1-globin* in both *zepo*-MO-injected and wild-type embryos at 24 hpf. However, the expression of α*A1-globin* and β*A1-globin* was reduced significantly in *zepo*-MO-injected embryos at 24 hpf.

4. Discussion

In this study, the *zepo* gene and its cDNA were cloned and characterized. The cDNA encodes a protein of 183 amino acids, which displays 55% and 32% identity to the fugu Epo and human Epo. RT-PCR analyses showed that *zepo* mRNA was primarily expressed in the heart and liver. Unlike the fugu Epo, zEpo contains two glycosylation sites and the recombinant zEpo was efficiently secreted into the culture medium as a glycoprotein. Knockdown of zEpo caused severe anemia leading to high mortality in zebrafish embryos and the expressions of specific markers for adult globin genes, such as α*A1-globin* and β*A1-globin*, were affected.

The genomic structure of *epo* genes from human and fugu have been determined [6]. In this study, the zebrafish *epo* gene consists of five exons and four introns spanning approximately 13.4 kb (Fig. 1). For comparison, the human *epo* gene spans only about 2.9 kb, while the fugu *epo* gene spans about 5.9 kb. The first intron of fugu and zebrafish *epo* gene is 3,105 bp and 5896 bp in length respectively, which is larger than that of human *epo* gene for only 584 bp in length. The second and the last introns of zEPO gene are also large, which are 2052 bp and 4735 bp in length, compared to those of human EPO gene with the length of 258 bp and 134 bp, respectively. Taken together, EPO genes from zebrafish, fugu and

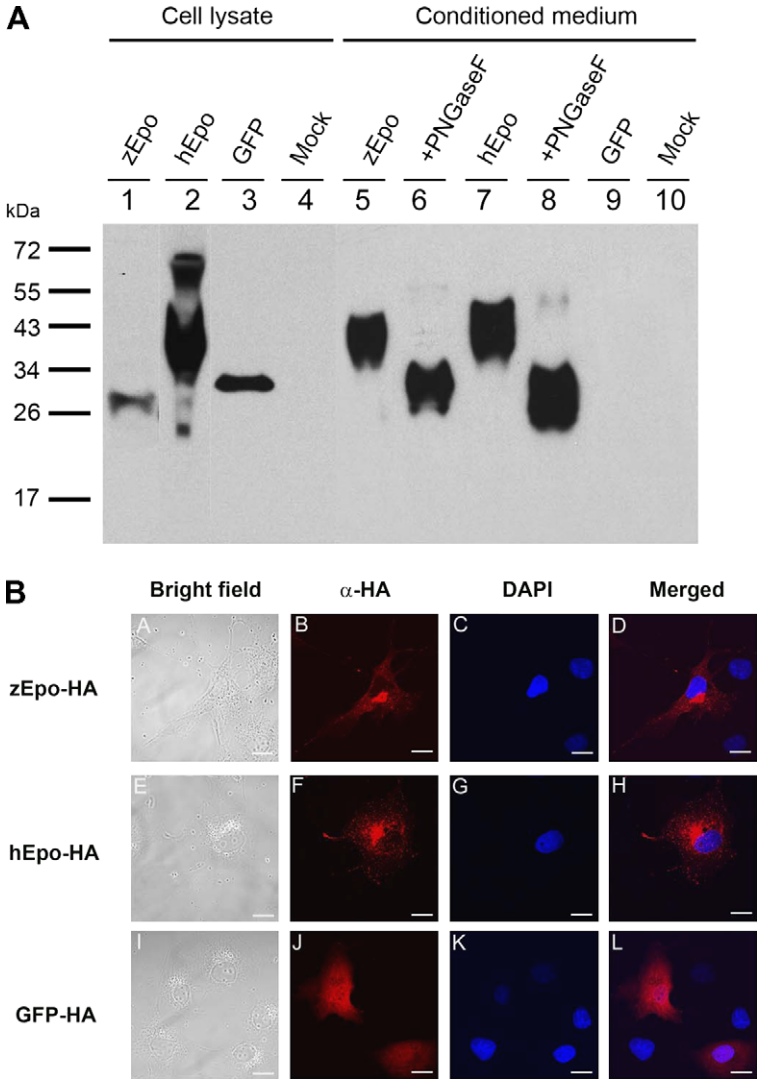

Fig. 3. Expression and cellular localization of zEpo protein in COS-1 cells. COS-1 cells were transfected with pCMV-zEpo-HA, pCMV-hEpo-HA or pCMV-GFP-HA as a control. (A) At 48 h post-transfection, the cell lysates (lanes 1–4) and the conditioned media (lanes 5–10) were collected for Western blot analysis. Untreated (lane 5 or 7) and *N*-glycosidase-treated (lane 6 or 8) culture supernatants displayed different electrophoretic mobility. (B) For immunofluorescence studies, transfected cells were immunostained with anti-HA antibody and Cy-3 conjugated anti-mouse antibody. The cell nuclei were stained with DAPI (blue). Bars, 10 μm.

human have identical exon-intron structure, but the sizes of introns are different.

The predicted amino acid sequence of zEpo exhibits an overall identity of 90%, 55% and 32% to the Epo from carp, fugu and human, which is consistent with the early report that fugu Epo shows 32% identity to human Epo [6]. The possible explanation for the higher identity of zEpo to carp Epo than to fugu Epo may be due to that carp and zebrafish belong to the same family of Cyprinidae, while fugu belongs to the family of Tetraodontidae. In the mammalian Epo family, they appear to be divergent, with the identity of human Epo to Epo from other mammals ranging from 80% to 91% [18]. The human Epo has four cysteine residues at positions 7, 29, 33 and 161 and these residues are conserved in all fish Epos (Fig. 1B). These residues form internal disulfide bonds that are essential for the biological activity of the recombinant human Epo [19,20]. Interestingly, the mouse Epo has proline at position 33, but has another cysteine at position 139 (Fig. 1B).

Human Epo is a glycoprotein and contains three N-linked sugar chains on Asn24, Asn38 and Asn83 as well as one O-linked sugar on Ser126 [7]. In the putative mature form of zEpo, two N-linked glycosylation sites, ^{38}NVT and ^{81}NQT, are found (Fig. 1B), while the fugu Epo has no N-linked glycosylation site [6]. In the COS-1 cell transfection experiment, the recombinant zEpo-HA protein was efficiently secreted into the culture medium as a glycoprotein and its sugar chains can be removed by the treatment of peptide-*N*-glycosidase F (PNGase F). The PNGase-treated zEpo appears to be slightly larger than the cytosolic zEpo (Fig. 3), suggesting that the secretary zEpo may contain the O-linked sugar chain. Human Epo has an O-linked sugar on Ser126 with the flanking sequences of TPPDAAS^{126}AA. The fugu Epo has no N-linked glycosylation site, but it contains an O-linked glycosylation site on Ser117 with the flanking sequences of TPPAS^{117}AA, which are homologous to those of human Epo [6] (Fig. 1B). On the other hand, the O-linked glycosylation site of zEpo is localized

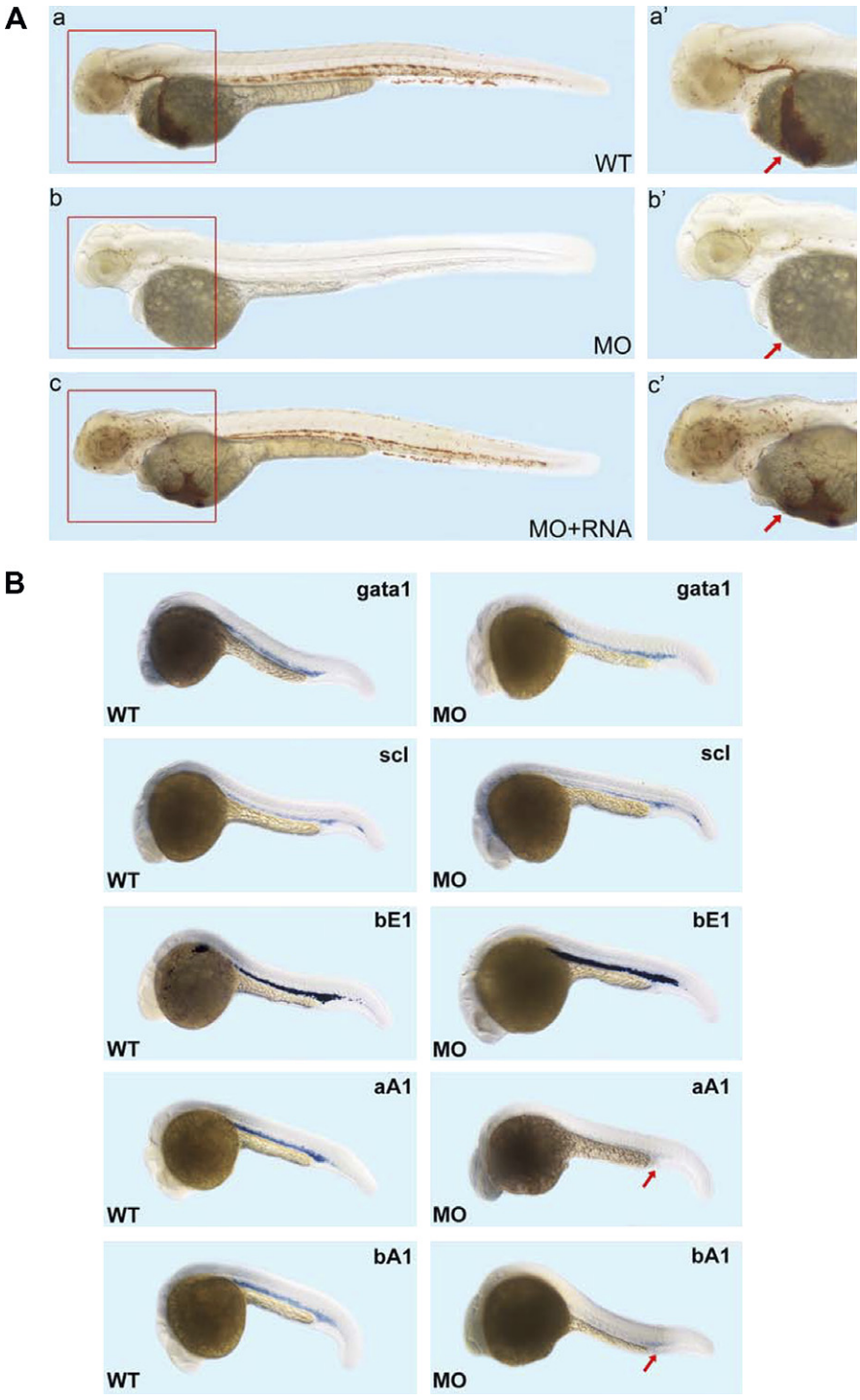

Fig. 4. Reduction of hemoglobin production and low expression of erythroid-specific genes in the *zepo*-MO-injected embryos. (A) *o*-dianisidine staining of wild-type (a, a′) or MO-injected (b, b′) embryos were performed at 48 hpf. The presence of hemoglobin (arrow) was significantly reduced in MO-injected embryos. However, the loss of hemoglobin in MO-injected embryos could be rescued by the injection of *zepo* RNA (c, c′). (B) Whole-mount in situ hybridizations with antisense *gata1*, *scl*, β*e1-globin* (bE1), α*A1-globin* (aA1) and β*A1-globin* (bA1) were performed. The images were taken from a lateral view, with anterior to the left and dorsal to the top. Embryos were stained under the same condition and for the same period of time to provide comparable sensitivity.

to the sequences of ^{114}PPTSSGED. The recombinant human Epo variant with either a double mutation (Gln38,83) or a triple mutation (Gln24,38,83) on the three N-glycosylation sites by replacing the asparagines with glutamines was secreted poorly from COS-1 and CHO cells [8]. Thus, whether the fugu Epo can be secreted efficiently in COS-1 cells or not needs further investigation.

Embryonic lethality has been observed in null mutations of the Epo gene or Epor gene in mice due to severe anemia and tissue hypoxia [21,14]. This phenotype is consistent with our

results that zebrafish embryos injected with *zepo*-MO displayed significant loss of hemoglobin as well as high mortality rate (Fig. 4). In the null mutations of the *JAK2* gene, an important tyrosine kinase in the Epo/Epor signal pathway, there is fewer primitive erythrocytes and no progression of definitive erythrocytes. Hemoglobinization of definitive erythroid cells is almost abolished in $JAK2^{-/-}$ fetal liver cells [22] but the expression of embryonic globins specific for the primitive erythrocytes appears less affected [23]. Similarly, in *zepo*-MO-injected zebrafish embryos, only the expression of adult globin genes, α*A1-globin* and β*A1-globin* was reduced significantly, while there was no change in the expression of embryonic globin gene, β*e1-globin* (Fig. 4).

Although the fugu *epo* gene was the first gene identified from non-mammalian vertebrate [6], the function and secretion ability of the fugu Epo has not been determined yet. In the present study, we isolated an *epo* gene from zebrafish and demonstrated that it is required for erythropoiesis using morpholino approach. Moreover, the recombinant zebrafish Epo protein can be efficiently secreted into the culture medium as a glycoprotein. Thus, this is the first fish Epo that has been expressed and extensively characterized.

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