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Review

Erythropoietins from teleosts

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Abstract. The epo genes of many teleosts, including zebrafish, have been cloned following the first identification of nonmammalian EPO from fugu in 2004. The zebrafish (Danio rerio) animal model is well suited for both developmental and genetic analyses for studying vertebrate erythropoiesis. The purpose of this review is to provide an update of recent progress in research on teleost EPO with a focus on its structure, expression and secretion. The EPO receptor and the downstream JAK/STAT signaling pathway are also discussed.

Keywords. EPO, EPO receptor, glycosylation, secretion, zebrafish.

Introduction

In mammals, erythropoietin (EPO) is an important glycoprotein hormone that functions as a principal regulator of erythropoiesis [1]. Effects of EPO are mediated by binding to the EPO receptor (EPOR), which is primarily expressed in hematopoietic progenitor cells [2]. Upon binding of EPO to its receptor, dimerization of EPOR occurs to activate the JAK/STAT signaling pathway [3]. Currently, recombinant human EPO has been used to treat anemia caused by chronic kidney disease [4] as well as to treat cancer patients with anemia induced by chemotherapy or radiotherapy [5, 6]. However, a number of studies in mammals suggest that EPO also plays non-erythropoietic functions in the central nervous system and other tissues. Therefore, recombinant human EPO has other therapeutic potentials in addition to its classical correction of anemia, such as the neuroprotective, renoprotective and cardio-protective potentials [7, 8]. Native human EPO was isolated in 1977 [9] and its gene cloned in 1985 [10]. After its sequence was determined, human EPO rapidly became used for treatment of anemia in renal failure [4]. The gene encoding the murine EPOR was cloned in 1989 [11]. It was 19 years after the cloning of the human *epo* gene that the first non-mammalian *epo* gene was cloned from fugu [12]. The cloning benefited from the disclosure of the fugu genomic draft [13]. Afterward, more teleost *epo* genes including zebrafish [14, 15] were also cloned. The zebrafish (*Danio rerio*) animal model is well suited for both developmental and genetic analyses for studying vertebrate erythropoiesis. This mini-review will update recent progress in research on teleost EPO specifically focusing on its structure, expression and secretion. The EPO receptor and the downstream signal JAK/STAT pathway are also discussed. It is important to elucidate the function of EPO-EPOR signaling in non-hematopoietic tissues

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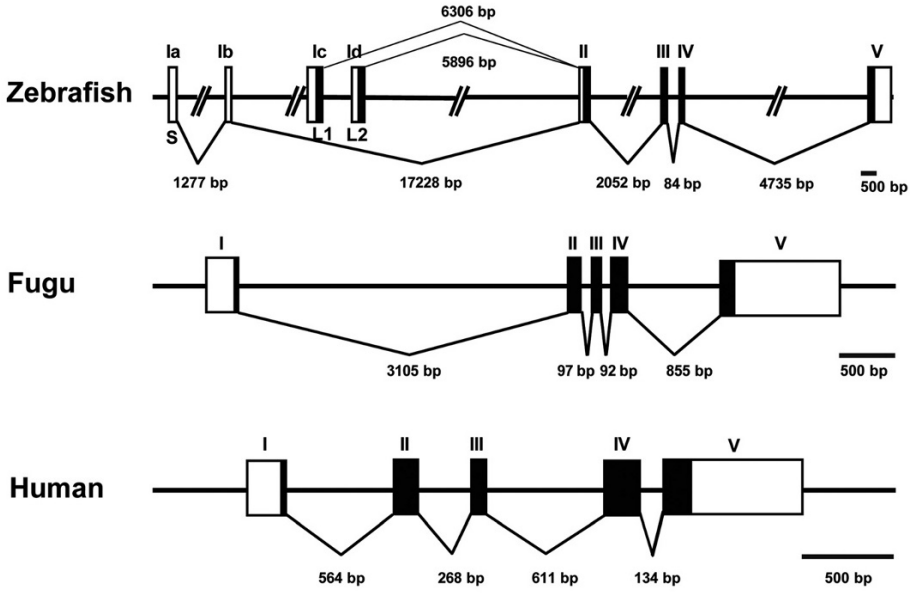

Figure 1. Genomic organization of the *erythropoietin* genes from zebrafish, fugu and human. Exons are indicated by the boxes numbered from 1 to 5, including the coding regions (solid boxes) and the untranslated regions (open boxes). Introns and the 5'-flanking regions are indicated by the solid lines. zEPO-S, zEPO-L1 and zEPO-L2 are encoded by exon 1a, exon 1b, exon 1c and exon 1d, respectively.

under normal or diseased conditions. The zebrafish system will be a powerful genetic model to accomplish such analysis of EPO signaling *in vivo*.

Structure

There is only one EPO protein in mammals [1]. However, in zebrafish we cloned three EPO-related transcripts, *zepo-L1*, *zepo-L2* and *zepo-S*, which are generated by alternative RNA splicing. In addition to the common C-terminal region of 154 amino acid residues, the N-terminal signal peptides of zEPO-L1, zEPO-L2 and zEPO-S contain 26, 23 and 17 amino acid residues, respectively. By transfection of each isoform into COS-1 cells, our results showed that both zEPO-L1 and zEPO-L2 were secretory glycoproteins, while zEPO-S was a cytosolic protein. Moreover, the secretion amount of zEPO-L1 present in culture medium was at least eight-fold more abundant than that of zEPO-L2.

Gene. The genomic structures of *epo* genes from human, fugu and zebrafish have been determined [12]. They consist of five exons and four introns spanning 2.9 kb (human), 5.9 kb (fugu) and 13.4 kb (zebrafish) (Fig. 1A). The first intron of fugu and zebrafish *epo* gene is 3,105 bp and 5,896 bp in length respectively, which is longer than that of the human *epo* gene (584 bp in length). Taken together, *epo* genes from human, fugu and zebrafish have identical exon-intron structure, but with different sizes of introns.

Through computational analysis, three EPO-related clones (accession nos.CF348767, DN903417 and CK239342) were found in the zebrafish EST databank, but their 5' end sequences are different, suggesting that they may be derived from alternative splicing. To confirm this, these cDNAs were used as a bait to perform an online BLAST search of the GenBank database and matched 8 non-contiguous regions in the 200556 bp zebrafish BAC clone DKEY-46E6. Comparison of this BAC clone to the sequences of each cDNA shows that all three *epo* cDNAs, termed *epo-L1*, *epo-L2* and *epo-S*, are contained within the putative eight exons and seven introns spanning approximately 25 kb (Fig. 1A). They have identical sequences derived from exon 2 to exon 5. However, the 5'-untranslated region (UTR) of *epo-S* is encoded by exon 1a and exon 1b, while the 5'-UTR and partial N-terminal region of *epo-L1* and *epo-L2* are encoded by exon 1c and exon 1d, respectively. These *epo* isoform sequences have been deposited in GenBank with the following accession numbers: EF426725 (*epo-L1*), EF426727 (*epo-L2*) and EF426726 (*epo-S*). A brain form of fugu *epo* has been reported to contain an alternative first exon coding for only four residues (MEFP) [12].

The deduced amino acid sequences of three zebrafish EPOs (zEPOs) (Fig. 2) were aligned to show that they shared a common C-terminal region of 171 amino acids, which covers the complete sequences of EPO-S. The N-terminal regions of EPO-L1 and EPO-L2 contain an additional 15 and 12 amino acid residues, respectively. The variable N-terminal region of each EPO isoform has significant effect on their secretion during protein synthesis; this will be discussed in the section of "Glycosylation and secretion".

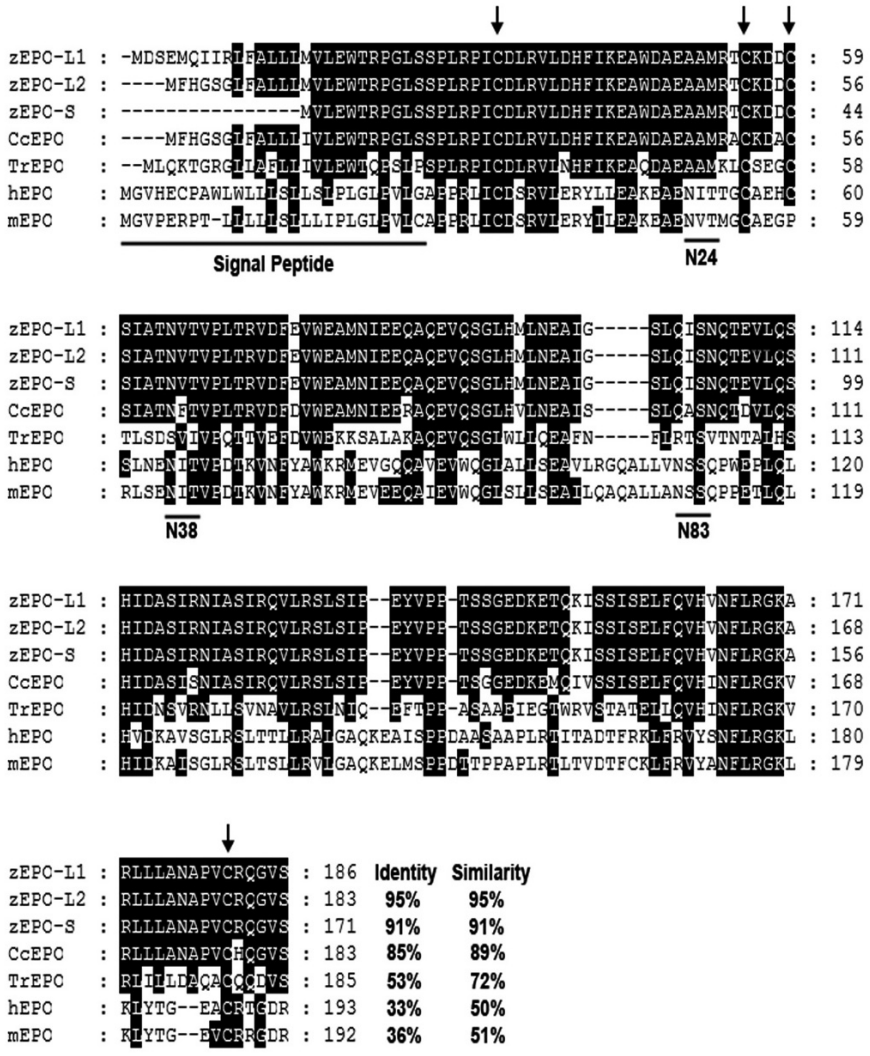

Figure 2. Alignment of amino acid sequences of three zebrafish erythropoietins with those from other species. Multiple alignment of erythropoietins from fish and mammals using CLUSTAL X: The amino acid sequence of three zebrafish erythropoietins (zEPO-L1; EF426725) (zEPO-L2; EF426727) (zEPO-S; EF426726) were 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.

Protein. The first *epo* gene from a non-mammalian vertebrate was cloned from fugu [12]. The predicted amino acid sequence of zEPO exhibits an overall identity of 90%, 55% and 32% to the EPO from common carp, fugu and human. The likely explanation for the higher identity of zEPO to carp EPO than to fugu EPO is that carp and zebrafish belong to the same family of Cyprinidae, while fugu belongs to the family of Tetraodontidae. There are four cysteine residues forming the internal disulfide bonds that are essential for the biological activity of mammalian EPO proteins [17, 18] also conserved in all fish EPOs (Fig. 2). The N-terminal 27 amino acid residues of the mature EPO form from zebrafish, common carp and fugu are almost identical except for a difference of three amino acids in fugu EPO (Fig. 2). Similar sequences are also found in the partial sequences of rainbow trout EPO (GenBank DQ288854).

Glycosylation and secretion. Prediction of the signal peptides of zebrafish EPO-L1 (zEPO-L1), zEPO-L2 and Tetraodon EPO (TrEPO) were shown in Figure 2. The putative signal peptides of zEPO-L1, zEPO-L2 and TrEPO contain 26, 23 and 25 amino acid residues, respectively, while the signal peptide of human EPO has 27 amino acid residues, one more residue compared to zEPO-L1.

The secretion efficiency of each isoform of zEPOs as well as TrEPO could be studied by transfection of each isoform into COS-1 cells. The cell lysates and culture medium were analyzed for protein expressions. Each EPO was labeled with hemagglutinin (HA) tag at the C-terminus to facilitate comparative analysis of EPO isoform expressions by Western blot. Interestingly, the amount of zEPO-L1-HA immunoreactivity present in culture medium was at least eight-fold more abundant than that of zEPO-L2-HA. Similarly, the amount of zEPO-L1-HA was more than that of zEPO-L2-HA in the cell lysate (Fig. 3). The molecular mass of zEPO-

L1-HA and zEPO-L2-HA were both reduced under treatment with peptide-*N*-glycosidase F (PNGase F) (Fig. 3), suggesting that these two isoforms undergo post-translational modification of glycosylation. As expected, zEPO-S-HA was found present only in the cell lysate due to a short signal peptide with a size of 17 amino acid residues. On the other hand, the presence of TrEPO-HA in both the cell lysate and culture medium suggests that its signal peptide is sufficient for secretion.

The putative mature form of zEPO contains two N-linked glycosylation sites, ^{38}NVT and ^{81}NQT (Fig. 2), whereas the fugu EPO has no N-linked glycosylation site [12]. In a transfection experiment, the recombinant zEPO-HA protein expressed in COS-1 cells was efficiently secreted into the culture medium as a glycoprotein. The sugar chains of the zEPO-HA can be removed by treatment with peptide-*N*-glycosidase F (PNGase F). The secretory form of zEPO treated with PNGase appears to be slightly larger than the cytosolic zEPO (Fig. 3), suggesting that the secretory zEPO may contain the additional 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 [12] (Fig. 2). On the other hand, the O-linked glycosylation site of zEPO is located at the sequences of ^{114}PPTSSGED. The recombinant human EPO variants with double (Gln38,83) or triple (Gln24,38,83) mutations on the three N-glycosylation sites by glutamine substitutions for the asparagines were secreted poorly from COS-1 and CHO cells [19]. However, the fugu EPO, which does not have N-linked glycosylation sites, can be secreted efficiently in COS-1 cells.

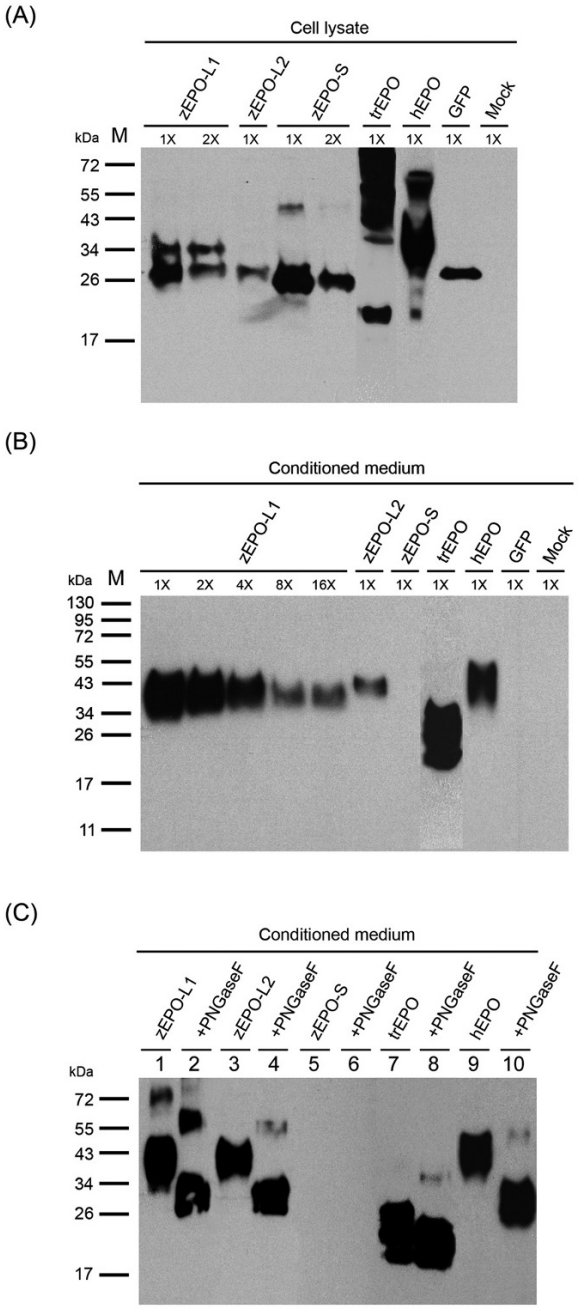

Figure 3. Expressions of various erythropoietins in COS-1 cells. COS-1 cells were transfected with pCMV-zEPO-L1-HA, pCMV-zEPO-L2-HA, pCMV-zEPO-S-HA, pCMV-trEPO-HA, pCMV-hEPO-HA or pCMV-GFP-HA as a control. The cell lysates (*A*) and the conditioned media (*B*) were collected for western blot analysis with anti-HA antibody at 48 h post-transfection. Samples were series diluted to the proper concentrations as indicated. (*C*) Culture supernatants of zEPO-L1-HA (8x diluted) zEPO-L2-HA, trEPO-HA and hEPO-HA were treated with N-glycosidase to display different electrophoretic mobility.

Expression and biological function

The mammalian EPO protein is mainly produced in the kidney. However, EPO is also produced by normal bone marrow cells [1]. In adult teleost, the production organ for various isoforms of EPO protein differed. For example, zebrafish *epo-L1*, *epo-L2* and fugu *epo* were predominantly expressed in heart and liver, whereas zebrafish *epo-S* was expressed in kidney. These data suggest that fish EPO proteins may have multiple functions at different sites in the body.

Expression. Kidney is the primary organ of EPO synthesis in adult mammals [1]. In adult zebrafish, the *zepo-L2* mRNA is predominantly expressed in heart and liver, while low expression is observed in the brain, gill, eye, intestine and kidney [14]. This expression pattern is consistent with that of fugu *epo* mRNA, which is predominantly expressed in heart and moderately so in liver and brain [12]. In addition,

the expression pattern of *zepo-L1* transcript is similar to that of *zepo-L2* mRNA, whereas the majority of the *zepo-S* mRNA is expressed in kidney (our unpublished data). The emergence of the bone marrow as a specialized erythropoietic tissue in mammals may be correlated with the change of the site of EPO production from heart to kidney [12]. Due to the lack of the bone marrow, most fish species such as fugu and zebrafish continue to use the heart as the site of EPO production. Interestingly, the major expression site of *zepo-S* mRNA is kidney, suggesting an advanced form of *zepo-S* that is secreted through a novel Golgi-independent pathway [20].

Both mammalian and fish EPO proteins are also synthesized in the adult liver and brain. Some recent studies in mammals indicate that EPO also plays non-erythropoietic functions, such as neuronal protection in the central nervous system [21].

Biological function. Embryonic lethality has been observed in null mutations of the *epo* or *epor* gene in mice due to severe anemia and tissue hypoxia [22, 23]. Zebrafish embryos injected with morpholino (MO) for zEPO-L2, zEPO-L2-MO, also displayed a significant loss of hemoglobin as well as a high mortality rate [14]. In mice, null mutations of the *jak2* gene, coding for an important tyrosine kinase in the EPO/EPOR signal pathway, result in fewer primitive erythrocytes and no progression of definitive erythrocytes. Hemoglobinization of definitive erythroid cells is almost abolished in *jak2*$^{-/-}$ fetal liver cells [24], but the expression of embryonic globins specific for the primitive erythrocytes appears to be less affected [25]. Similarly, in zEPO-L2-MO-injected zebrafish embryos, only the expression of adult globin genes, *αA1-globin* and *βA1-globin* was reduced significantly, while there was no change in the expression of embryonic globin gene, *βe1-globin* [14].

In mammals, the major function of EPO is to regulate erythropoiesis via its induction under the stress of anemia and hypoxia. Similarly, zEPO expression is increased two-fold in hypoxia-treated hearts compared to untreated controls [15]. An increase of EPO protein concentration in rainbow trout kidney has also been detected by using a polyclonal antibody raised against a fish-specific peptide [26]. These findings demonstrate the conserved responses of fish and mammalian erythropoietic systems to hypoxia [27]. It has been characterized that hypoxia induces EPO production through transcriptional regulation by hypoxia-inducible factor (HIF) in mammals and fish [27, 28].

EPO receptor and downstream signal transduction pathways

The function of EPO is mediated through its binding to EPOR. The EPO:EPOR engagement leads to dimerization of EPOR, as well as to the activation of the JAK/STAT signaling pathway [3]. So far, the EPO signaling pathway has been primarily investigated in mammals, but knowledge of EPOR and its function via the JAK/STAT pathway remains scarce in fish.

EPO receptor. EPO receptors belong to the type I cytokine receptor family with a single transmembrane domain. They form homodimers in the presence of their respective ligands [29], associate exclusively with JAK2, and signal through the activation of STAT5 [30].

The zebrafish *epor* gene has been cloned, and the encoded protein has an overall identity of 22 % and 27 % in comparison with the EPOR from *Xenopus laevis* and human. However, it shares slightly higher identities with those of other teleost species: 44 % with fugu, and 41 % with *Tetraodon nigroviridis* [15]. Zebrafish EPOR possesses some conserved features characteristic of vertebrate EPORs. For example, 1) the extracellular domain contains two pairs of cysteines in the N-terminal subdomain and the WSXWS motif in the C-terminal subdomain; 2) highly conserved box 1 and box 2 regions are found within the intracellular domain of EPOR and, 3) some tyrosine residues in the distal cytoplasmic domain that are phosphorylated after EPO stimulation and act as docking sites for downstream signaling molecules are also conserved.

In mammals, loss of EPOR causes a slight effect on the primitive erythrocyte number, but results in a complete block in definitive erythropoiesis [23]. Similarly, injection of zebrafish EPOR-MO into zebrafish embryos can cause a modest decrease in primitive erythrocyte number at 36 hour post-fertilization (hpf) and a complete block in definitive erythropoiesis with the absence of erythrocytes at four days post-fertilization (dpf) [15]. In adult zebrafish, the *zepor* mRNA is expressed in brain, heart, kidney, liver and spleen [15]. This expression pattern is a partial match with that of *zepo* mRNA, which is predominantly expressed in heart and moderately so in liver and brain, as mentioned previously.

Janus kinase family. The *Janus* kinase (JAKs) family belongs to the non-receptor protein tyrosine kinase family and is presently comprised of four members in mammalian species, JAK1, JAK2, JAK3, and TYK2. The JAK1, JAK2 and TYK2 are expressed ubiqui-

tously in many tissues whereas JAK3 is expressed primarily in hematopoietic cells [31–33].

The complete genomic DNA sequences, including the promoter regions of the *jak1*, *jak2*, *jak3* and *tyk2* genes from *Tetraodon fluviatilis* (also known as *Tetraodon nigroviridis*), have been determined [34, 35]. Tetraodon JAK1 and JAK2 display higher amino acid identity of 59.5% and 66.8%, respectively, than those of human JAK1 and JAK2, whereas Tetraodon JAK3 and TYK2 show lower amino acid identity to their mammalian counterpart with 47.6% and 45.5% identity, respectively [34, 35]. Other fish JAK1 homolog has been cloned and characterized from common carp [36] and zebrafish [37]. The complete sequence of JAK3 is also cloned from common carp [38].

It is interesting to note that there are two *jak2* genes in zebrafish genome, but their temporal expressions during development are different [39]. JAK2a is expressed in erythroid precursors and plays very important roles in early erythropoiesis. In contrast, JAK2b is expressed in the developing lens and nephritic ducts, but not in hematopoietic tissue. The total amino acid sequences of zebrafish JAK2a show 65% identity to human JAK2, compared to 66.8% identity between Tetraodon JAK2 and human JAK2. Currently, only partial sequences of zebrafish JAK2b are known. The existence of two *jak2* genes in zebrafish genome is proposed to result from an early duplication specific to ray finned fish.

The role of JAK2a in zebrafish hematopoiesis has been investigated by MO-knockdown and overexpression of its constitutively active form. Injection of zebrafish JAK2a-MO into zebrafish embryos resulted in the reduction of erythroid cells and STAT5 phosphorylation, whereas injection of the constitutively active form of JAK2a increased erythropoiesis [40].

STAT family. Signal transducers and activators of transcription (STATs) are a family of latent cytoplasmic transcription factors that are rapidly activated in response to various extracellular polypeptide ligands such as cytokines, growth factors and hormones [41]. The activation of STAT is an evolutionarily conserved mechanism. In mammals, there are seven distinct STAT proteins [42], while only one related molecule has been found in *Drosophila* [43]. Among mammalian STATs, STAT1 is critical for interferon function as well as innate immunity [44], while STAT3 is required for interleukin-6 (IL-6) signaling in hematopoietic cells as well as anti-apoptosis [45, 46]. In addition, STAT5a and STAT5b have been shown to play important roles in growth, lactation and hematopoiesis [47, 48]. In contrast to the broad range of biological effects derived from STAT1, STAT3, and STAT5, STATs 2, 4, and 6 have relatively restricted functions, centered on immune response regulation. STAT2 is activated only by α/β interferon, STAT4 by IL-12 and STAT6 by IL-4 and IL-13 [49,50].

In fish, STAT1 and STAT3 from zebrafish were first to be cloned and characterized [51]. Zebrafish STAT1 displays an overall identity of 63.9% to mouse homologue and can substitute for mammalian STAT1 to support the survival of STAT1-deficient U3A human cell line. On the other hand, zebrafish STAT3 is highly conserved to human STAT3 (86.5% identity), and its gene is expressed in the central nervous system in a similar manner to the mammalian system. Another fish STAT1 homolog has been cloned and characterized from crucian carp, *Carassius auratus* [52].

The fish STAT5 gene has been isolated and characterized from Tetraodon [53]. This gene is composed of 19 exons spanning 11 kb. The full-length cDNA of Tetraodon *stat5* (Tn*stat5*) encodes a protein of 785 amino acid residues. From the amino acid sequence comparison, TnSTAT5 is most close to human STAT5a and STAT5b with an overall identity of 76% and 78%, respectively. As reported previously, a chimeric STAT5 was generated by fusion of the kinase-catalytic domain of carp *Janus* kinase 1 (JAK1) to the C-terminal end of TnSTAT5. The fusion protein was expressed and was tyrosine-phosphorylated by its kinase domain. The fusion protein exhibits specific DNA binding and transactivation potential towards an artificial fish promoter as well as authentic mammalian promoters such as the β–*casein* promoter and the cytokine inducible SH2 containing protein (CIS) promoter when expressed in both fish and mammalian cells [53].

Recently, two zebrafish STAT5 orthologues, STAT5.1 and STAT5.2, have been cloned [54]. Furthermore, the constitutively active form of zebrafish STAT5.1 was generated based on the previously identified murine STAT5a mutants, and its overexpression leads to an increase in the number of hematopoietic cells of the myeloid, erythroid, and B-cell lineage [55]. On the other hand, injection of zebrafish STAT5.1-MO into zebrafish embryos resulted in anemia [15]. These data suggest that STAT5 is required for zebrafish erythropoiesis.

Conclusion

The presence of the EPO-EPOR system in a variety of tissues, including brain, heart, and kidney, indicates that EPO has other activities beyond its known regulation of red blood cell production. Currently,

recombinant human EPO has other therapeutic potentials in addition to its classical correction of anemia, such as the neuroprotective, renoprotective and cardio-protective potentials. An evaluation of the function of EPO-EPOR signaling in non-hematopoietic tissues under normal or diseased conditions is required. Therefore, the zebrafish system will be a powerful genetic model to accomplish such analysis of EPO signaling *in vivo*.

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