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Expression of Erythropoietin Receptor–Like Molecule in *Xenopus laevis* and Erythrocytopenia upon Administration of Its Recombinant Soluble Form

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The regulation of hematopoiesis in non-mammalian vertebrates is poorly understood. This is partly because the structures and effects of most hematopoietic regulators have not been identified. As a first step towards studies on the key mechanism of hematopoietic regulation among phyla as well as the diversity of organisms, we have focused on amphibian hematopoiesis. A cDNA sharing the highest degree of homology with mammalian erythropoietin (EPO) receptors, tentatively named *xl*EPOR, was cloned from a cDNA library of *Xenopus laevis* immature erythrocytes. The comparative identities of the deduced entire amino acid sequence to mammalian EPO receptors were quite low, although functional domains indispensable for erythropoietic activities were found in the molecule. Northern analysis revealed that *xl*EPOR were expressed in peripheral blood cells. In the peripheral blood of phenylhydrazine-treated adult *Xenopus*, immature erythrocytes expressing *xl*EPOR were identified by *in situ* hybridization and immunostaining with polyclonal antibodies to *xl*EPOR. To confirm the biological functions of this molecule, the extracellular domain of *xl*EPOR (*i.e.*, soluble *xl*EPOR) was administered to adult *Xenopus* by consecutive intracardiac injection. The peripheral erythrocyte counts were decreased gradually; meanwhile, immature erythrocytes appeared in the circulation, demonstrating that *xl*EPOR plays a significant physiological role in erythropoiesis in *Xenopus laevis*.

Key words: erythrocyte, erythropoieisis, erythropoietin receptor, soluble receptor, *Xenopus*.

Abbreviations: dDPBS, 7/9 diluted Dulbecco's phosphate buffered saline; EPO, erythropoietin; EPOR, EPO receptor; MGG, May-Grünwald-Giemsa; PHZ, phenylhydrazine; soluble *xl*EPOR, extracellular domain of *xl*EPOR; TB, toluidine blue; *xl*EPOR, *Xenopus laevis* EPOR; *xl*EPOR PoAb, anti-*xl*EPOR polyclonal IgG; *xt*EPOR, *Xenopus tropicalis* EPOR.

Erythropoiesis is understood to be one of the elemental systems of homeostasis in most vertebrates. Erythropoietin (EPO) and EPO receptor (EPOR) carry out essential roles in erythropoiesis by promoting the proliferation, differentiation and survival of erythrocyte progenitors, including burst-forming units-erythroid (BFU-E) and colony-forming units-erythroid (CFU-E), a process that is triggered by the binding of EPO to EPOR on the cell surface. The number of EPOR is maximized at stages from CFU-E to pronormoblasts, and subsequently it decreases gradually such that the response to EPO finally diminishes (*1*, *2*). In response to hypoxia, EPO is produced by the kidney and, to a lesser extent, by the liver, and released into the blood stream. Several cDNA and genomic sequences of mammalian EPO and EPOR have been determined, and their phylogenetical relationships elucidated (*3*–*7*).

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In non-mammalian vertebrates, the physiological mechanisms of erythropoiesis and related molecules have not been well clarified. In the case of EPOR, it has been reported that chicken erythroblasts with genetically introduced murine EPOR respond to both anemic chicken serum and recombinant human EPO, suggesting that endogenous EPO and EPOR homologues exist in the chicken (*8*). EPO-like molecules bound to anti human EPO antibody have also been found in non-mammalian sera (*9*). In *Xenopus laevis*, as is the case with mammalian species, thrombopoietin (TPO)/c-MPL signaling in erythropoiesis at an early stage of the developing *Xenopus* embryo has recently been reported (*10*). Nevertheless, to date, only limited studies have been conducted to characterize molecules relating to erythropoiesis in non-mammalian species. Recently, the EPO gene of teleost fish, *Fugu rubripes*, was identified from a draft database of its genome sequence (*11*), and several candidate genes for EPO and EPOR in non-mammalian species (zebrafish, *Tetraodon nigroviridis*, *Fugu rubripes* and *Xenopus tropicalis*) were identified in online genomic resources including the Ensembl (http://www.ensembl.org/) and National Center for Biotechno-

logy Information (NCBI; http://www.ncbi.nlm.nih.gov/). Their putative EPO and EPOR proteins share low identity values compared to those of mammals. Conversely, the biological characterization of EPO- and EPOR-like molecules found in the databases of non-mammalian species has not yet been achieved.

In *X. laevis,* peripheral blood cells have a particular morphology compared to mammals (*12*). Mature circulating blood cells, erythrocytes that confer oxygen delivery and thrombocytes participating in hemostasis, are nucleated. As yet it remains unclear, however, what mechanisms exist, and which cytokines are involved in the regulation of definitive erythropoiesis in adult *Xenopus laevis*. We report here the identification of a *X. laevis* derived EPOR-like molecule, tentatively named *xl*EPOR, that is specifically expressed in immature erythrocytes. Its primary function in adult *Xenopus* was then investigated. Accordingly it was revealed that *xl*EPOR has a significant biological role in definitive erythropoiesis in *Xenopus*, since erythrocytopenia is induced by the administration of recombinant soluble *xl*EPOR. This work emphasizes *X. laevis* to as a practical new animal model for the study of erythropoiesis.

MATERIALS AND METHODS

cDNA Cloning of X. laevis EPOR Like Molecule—Male African clawed frogs, *X. laevis* (20–30 g body weight) were purchased from Aquatic Animal Supply (Misato, Saitama, Japan), and induced to anemia by intraperitoneal injection of 25 mg/kg of phenylhydrazine (PHZ) on day 0. After 10 days, peripheral blood cells were collected by cardiac puncture from one of the PHZ injected animals. Blood samples were overlaid onto discontinuous Percoll (Amersham Biosciences Corp., NJ) gradients [90, 60, 50, 40% (v/v)] buffered with 7/9 diluted Dulbecco's phosphate buffered saline (dDPBS; KCl 0.155 g, Na_2HPO_4–$12H_2O$ 1.80 g, NaCl 6.22 g, KH_2PO_4 0.155 g, pH 7.3 per liter) and centrifuged at 500 × *g* for 15 min at room temperature to separate immature erythrocytes. Immature erythrocytes were washed three times with dDPBS and verified by May-Grünwald-Giemsa (MGG) staining. Their total RNA and poly(A)$^+$ RNA were extracted using commercial reagents, ISOGEN (NIPPON GENE, Tokyo, Japan) and Oligotex-dT (TAKARA BIO Inc., Otsu, Japan), respectively. An immature erythrocyte cDNA library in a λZAP II vector (Stratagene, LaJolla, CA) was prepared using a Timesaver cDNA synthesis kit (Amersham) and Gigapack III packaging extract (Stratagene) according to the instruction manuals. A library of approximately 1.2×10^6 clones was obtained and amplified once, and then served for screening.

To prepare a cDNA probe for screening, the Expressed Sequence Tag (EST) sequence of the putative *X. laevis* EPOR (*xl*EPOR) was obtained from GenBank through the web pages of NCBI (GenBank accession number: BM180725) by Basic Local Alignment Search Tool (BLAST) searching with human EPOR protein sequence using the "tblastn" algorithm. To generate the *X. laevis* EPOR cDNA fragment, RT-PCR on *Xenopus* immature erythrocyte total RNA was performed using *xl*EPOR Fw1 (5′-ACGCGTGGGTGCTGATATGC-3′) and *xl*EPOR Re2 (5′-TGAAAGCCATGTCTGAAGATCCC-3′) primers. The obtained cDNA fragment was cloned once into pGEM-T Easy vector (Promega Corporation, WI), and its nucleotide sequence was verified by using a DNA sequencer (dNA sequencer Model 4000L, LI-COR, Lincoln, NE). The cDNA fragment was labeled with biotin-16-dUTP (Roche Diagnostics GmbH, Mannheim, Germany) by a PCR technique for use as a probe for screening the cDNA library. To isolate cDNA clones from the cDNA library, we applied a plaque hybridization technique. The biotinyled probe was detected by incubation with streptactin conjugated alkaline phosphatase (BioRad Laboratories, Richmond, CA), washed three times with Tris buffered saline (TBS; 20 mM Tris-HCl pH 7.5, 500 mM NaCl), and developed with 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt (BCIP)/Nitro-TB (Dojindo Co. Ltd., Kumamoto, Japan). The nucleotide sequences of the obtained clones were determined by sequencing from both directions. The signal peptide of *xl*EPOR was predicted based on signalP programs (*13*), and compared with the reported amino acid sequences of other mammalian species.

Expression of xlEPOR mRNA—To explore *xl*EPOR mRNA expression in adult tissues, northern hybridization was performed. We amplified a cDNA fragment (1,615 bp) covering the entire coding region of *xl*EPOR and labeled it with biotin-16-dUTP (Roche). Total RNA (10 μg) from various tissues and mRNA (1 μg) from normal and immature erythrocytes were separated in 1% (w/v) agarose gels under denaturing conditions, and then blotted onto Hybond-N$^+$ (Amersham). The blotted RNAs were then hybridized with the biotinyled probe. After stringent washings, a hybridized probe was detected by incubation with streptactin conjugated alkaline phosphatase, followed by a CDP-Star chemiluminescence reagent (Amersham). Chemiluminescence signals were detected by exposure to X-ray film.

Normal and immature erythrocyte–rich peripheral blood cells were obtained and cytocentrifuged on glass slides, fixed with 4% (w/v) paraformaldehyde for 20 min, and incubated with 0.1% (v/v) Triton X-100 for 5 min. Subsequently, *in situ* hybridization of the cells was performed. Antisense *xl*EPOR and *Xenopus gata*-1A (erythroid marker) (*14*) RNA probes were transcribed *in vitro* in the presence of biotin-16-UTP (Roche). After washing twice with TBS followed by 2× SSC, the slides were prehybridized with hybridization buffer [50% (v/v) formamide, 0.1% (w/v) Ficoll 400, 0.1% (w/v) bovine serum albumin, 0.1% (w/v) polyvinylpyrroridon, 5× SSC, 250 μg/ml yeast tRNA, and 170 μg/ml salmon sperm DNA] for one hour at 37°C. Then biotinyled RNA probes in hybridization buffer were added, and the slides were incubated overnight at 37°C. After extensive washing, the biotinyled probes were detected with BCIP/Nitro-TB.

In Vivo Administration of Soluble xlEPOR—The cDNA fragment encoding the extracellular domain of *xl*EPOR (soluble *xl*EPOR, 1–216 a.a.; Fig. 2A) was amplified by PCR and inserted into a pGEX-6P-1 vector (Amersham). Glutathione S-transferase (GST) and soluble *xl*EPOR fusion protein (GST-soluble *xl*EPOR) were expressed in *Escherichia coli*. Since the majority of the GST-soluble *xl*EPOR (52 kDa) was insoluble, insoluble proteins were denatured once in a denaturing buffer (6 M urea, 5 mM EDTA and 10 mM DTT in DPBS) after extensive washing. The soluble protein fraction obtained after centrifu-

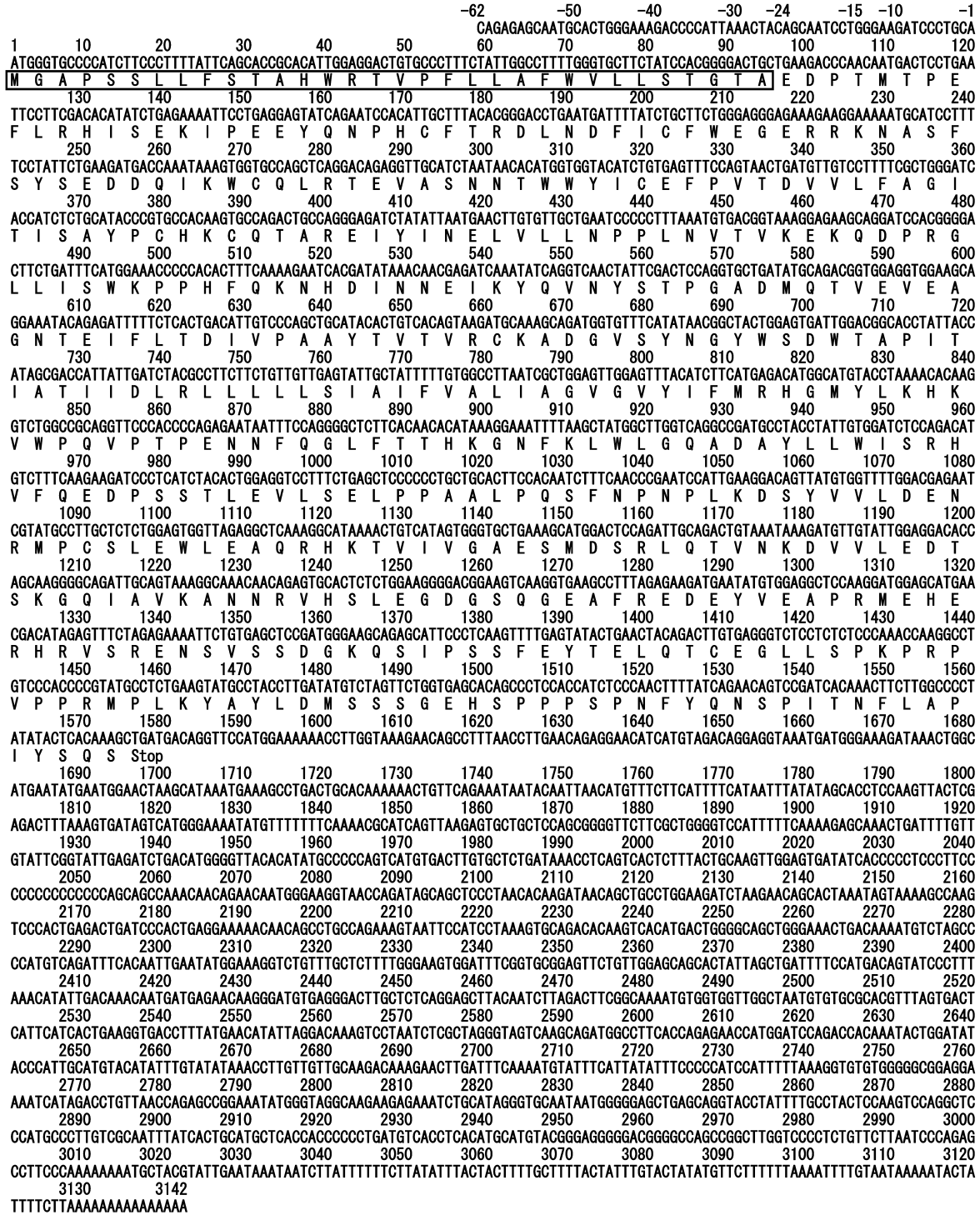

Fig. 1. **cDNA sequence and deduced amino acid sequences of *xl*EPOR.** The sequence of the *Xenopus laevis* EPOR is 3,204 bp in length. It contains a coding sequence of 1,578 bp, a 5′ UTR of 62 bp and a 3′ UTR of 1,564 bp. The 3′ UTR has a polyadenylation signal (AATAAA), followed by a poly(A)+ tail (15 adenines). The deduced amino acid sequence is 525 residues in length. The predicted signal peptide determined as described in the text is boxed.

gation was then subjected to a series of dialyses to remove DTT and urea. The refolded protein fraction was applied onto a Glutathione Sepharose 4B column (Amersham), and purified GST-soluble *xl*EPOR was obtained. The protein was then digested with PreScission protein protease (Amersham), dialyzed, and reapplied to the column to remove GST and the protease. The extracellular domain of *xl*EPOR has seven cysteine residues; to exclude the possibility that the purified protein might contain incorrectly folded molecules, and thus not have full activity, a high dose of purified soluble *xl*EPOR (250 μg/kg body weight) in dDPBS was injected into male

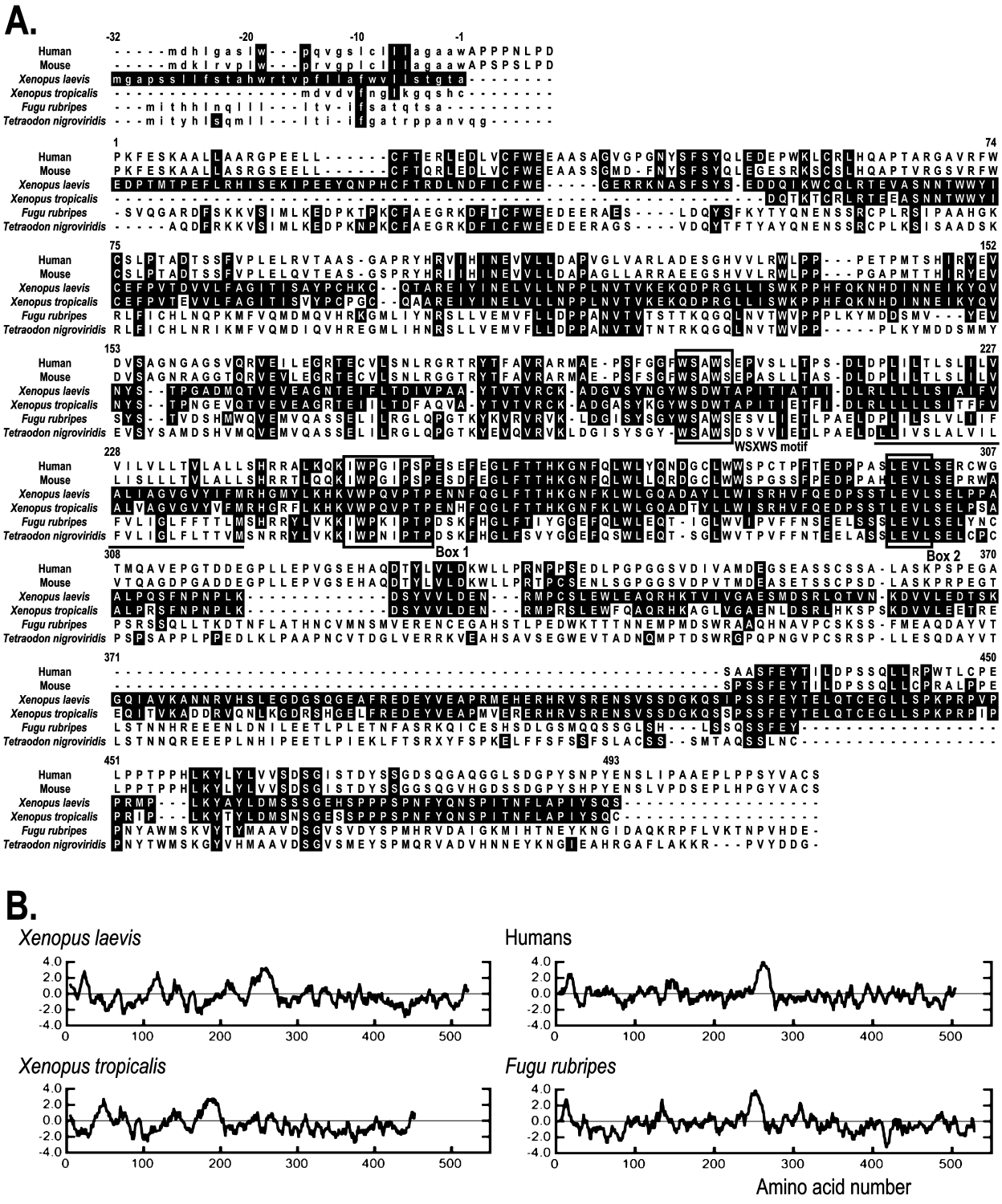

Fig. 2. **Homology of EPORs from various vertebrate species.** A: Amino acid sequence homology of putative mature EPORs between humans, mice, *Xenopus laevis*, *Xenopus tropicalis*, *Fugu rubripes* and *Tetraodon nigroviridis*. Signal peptide sequences are indicated in lower case letters. The numerical orders of the amino acid residues start at the mature proteins of *xl*EPOR. The amino acid residues which are identical to *xl*EPOR are shaded. Putative transmembrane domains are underlined. The WSXWS, box1 and box2 motifs are boxed. B: Hydropathy plots for EPORs from *X. laevis* (*xl*EPOR), *X. tropicalis* (*xt*EPOR), humans and *F. rubripes* generated using Kyte and Doolittle hydrophilicity parameters.

Xenopus every other day by intracardiac injection. Peripheral blood was collected by heart puncture with an EDTA-2Na coated capillary every four days. The blood cells of each individual were stained with crystal violet and each blood cell type (erythrocyte, leukocyte, and thrombocyte) was microscopically counted on an improved

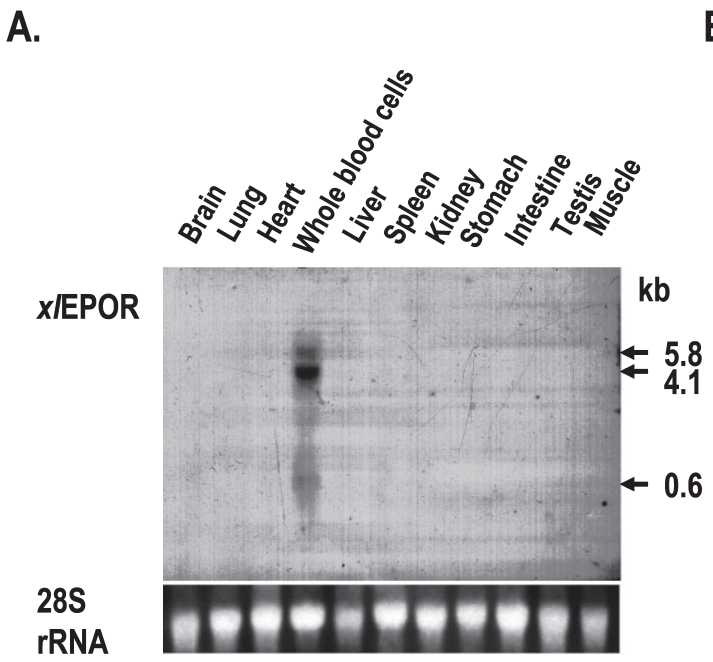

Fig. 3. **Expression of *xl*EPOR mRNA in various tissues from adult *Xenopus laevis*.** A, B: northern hybridization with biotinyled *xl*EPOR cDNA probe were performed. Total RNA (10 μg) from various tissues (A) and mRNA (1 μg) from mature and immature erythrocytes (B) were used. The 28S ribosomal RNA were stained with ethidium bromide to check RNA quality. The sizes of the mRNA bands are indicated at the side of the figures.

Neubauer counting chamber. For morphological studies, 1.0×10^5 blood cells were cytocentrifuged on glass slides. After staining with MGG and toluidine blue (TB) reagents, mature and basophilic immature erythrocytes were counted. Blood cell typing on the slides was conducted according to the method described in reference (*12*). All animal experiments in this study were conducted according to the Regulations for Animal Experimentation at Waseda University.

Immunostaining—Anti-*xl*EPOR rabbit antiserum was obtained by immuning rabbit with soluble *xl*EPOR purified as described above. The anti-*xl*EPOR polyclonal IgG (*xl*EPOR PoAb) was prepared by Prosep-G column chromatography (Millipore Corp. MA), and the specificity of *xl*EPOR PoAb was confirmed by western blotting against soluble *xl*EPOR. The biotinyled *xl*EPOR PoAb was prepared using Biotin-$(AC_5)_2$-OSu (Dojindo) according to the instruction manual, and immunostaining of the blood cells was performed. Blood cells on the glass slides were fixed with formalin-acetone [1.4 mM Na_2HPO_4, 7.3 mM KH_2PO_4, 45% (v/v) acetone, 25% (v/v) formalin], and washed three times with deionized water, followed by three times with TBS. Then the cells were incubated at 4°C for one hour in TBS containing 4% (w/v) Block Ace (Yukijirushi, Sapporo, Japan) (4% BA/TBS) to avoid nonspecific protein binding. After washing three times with TBS, 20 μg/ml of the biotin labeled *xl*EPOR PoAb in 0.4% BA/TBS was added; the samples were incubated for one hour, and then washed three times with TBS. Streptavidine-conjugated Alexa Fluor 568 fluorescent (Invitrogen, Carlsbad, CA) was added, and the samples were incubated for 1 h After washing three times with TBS, the nuclei were counterstained with TO-PRO-1 (Invitrogen). Images were acquired on a Leica TCS SPII confocal laser scanning microscope (Leica, Heidelberg, Germany).

RESULTS

cDNA Cloning of xlEPOR—The PCR product (407 bp) obtained with the *xl*EPOR primers was biotinyled and used to screen the immature erythrocyte cDNA library. Three clones encoding putative *xl*EPOR were obtained from 1×10^6 clones. One of the clones, F51, was finally analyzed further (Fig. 1). We searched for homologous molecules having similar amino acid sequences to *xl*EPOR on the BLAST server in NCBI and Fast Alignment Search Tool Algorithm (FASTA) server in the DNA Data Bank of Japan (DDBJ). Since we have not been able to identify other homologous molecules in *X. laevis*, we tentatively named this molecule *X. laevis* EPOR (*xl*EPOR). Mammalian EPOR molecules exhibit slightly higher homology identity values with *xl*EPOR, and some molecules, including cytokine receptor super family, such as c-mpl, leptin receptor and prolactin receptor, have lesser homology identity values.

The identities of the entire deduced amino acid sequences of *xl*EPOR with human and murine EPOR molecules are 33.3% and 34.2%, respectively. These values are much lower identities among mammalian EPOR sequences (more than 80%). Based on the nucleotide sequence, the identities of the sequences were found to be less than 50% between *Xenopus* and mammals. When we compared *xl*EPOR amino acid sequences to those of non-mammalian EPOR homologues obtained from Ensemble and DDBJ genome databases (Fig. 2A), the putative *Xenopus tropicalis* EPOR (*xt*EPOR; Ensembl Genscan accession ID: GENSCAN00000091844) has high identity values (84.5%), while the putative *Fugu rubripes* EPOR (Ensembl accession ID: SINFRUP00000143529, 26.9%) and the putative *Tetraodon nigroviridis* EPOR (Ensembl accession ID: AY374481, 32.2%) have lower values than that of *X. tropicalis*.

Although the homology identity values of entire amino acid sequences between *xl*EPOR and mammal EPOR are low, certain functional domains indispensable for exert-

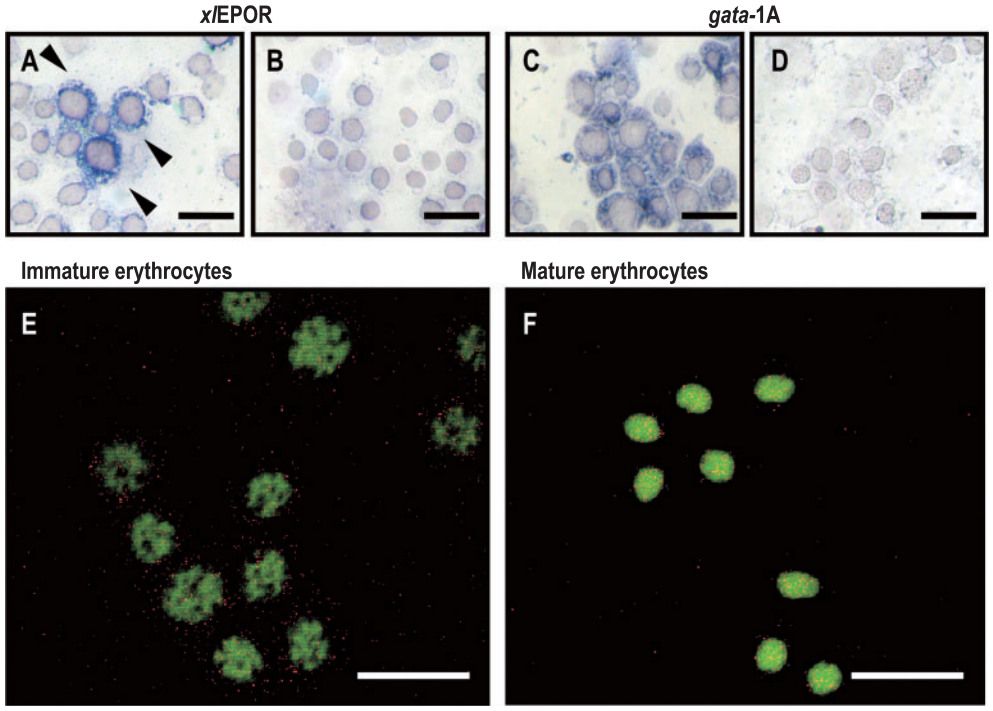

Fig. 4. ***xl*EPOR mRNA is expressed in immature erythrocytes and the *xl*EPOR protein is localized to their surface membranes.** A–D: *in situ* hybridization analysis was performed with biotinyled *xl*EPOR and *gata*-1A RNA probes. A: *xl*EPOR antisense RNA probe; immature erythrocytes with large nuclei were stained (arrowhead), while mature erythrocytes were not. B: *xl*EPOR sense RNA probe; negative control. C: *gata*-1A antisense RNA probe; immature erythrocytes with large nuclei were stained, while mature erythrocyte were weakly stained. D: *gata*-1A sense RNA probe; negative control. E, F: immunostaining with *xl*EPOR PoAb was performed. The biotinyled *xl*EPOR PoAb was detected by streptavidine conjugated Alexa Fluor 568 fluorescent (red). The nuclei were counterstained with TO-PRO-1 iodide (green). E: immature erythrocytes in anemic peripheral blood; *xl*EPOR proteins were localized to the cell surface membranes of immature erythrocytes (red). F: mature erythrocytes in peripheral blood. Bars represent 20 μm.

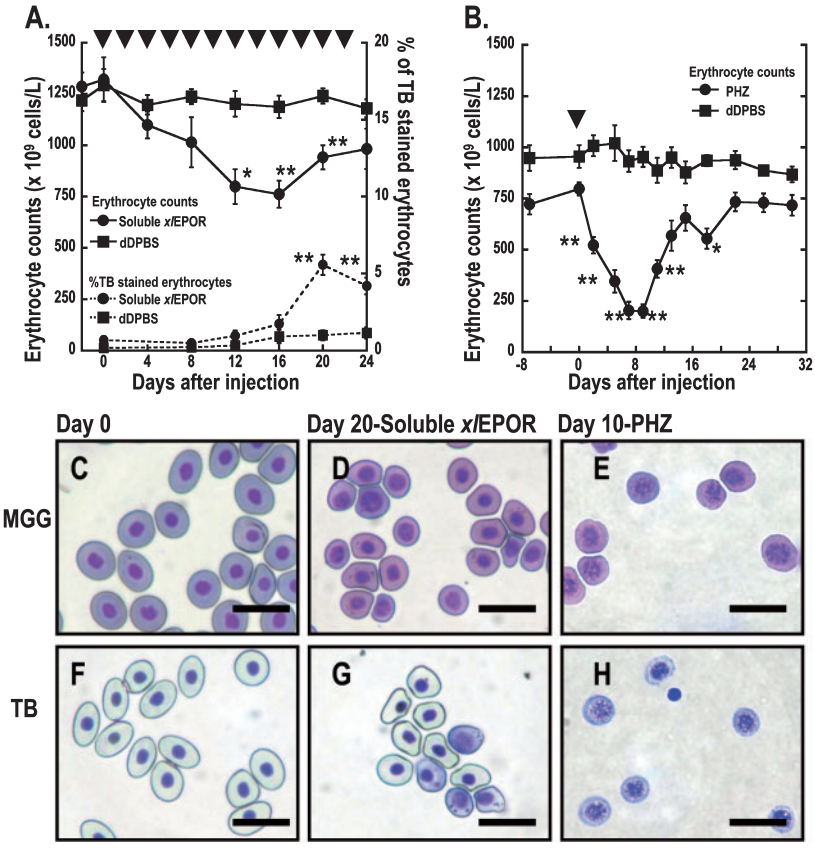

Fig. 5. **Effect of soluble *xl*EPOR on peripheral erythrocyte counts in *Xenopus laevis*.** A: Erythrocyte counts (solid lines) and percent of TB stained erythrocytes (dotted lines) in peripheral blood after the administration of soluble *xl*EPOR. Soluble *xl*EPOR was intracardially injected at 250 μg/kg body weight every other day (arrowhead). Closed circles, soluble *xl*EPOR-treated animals; closed squares, controls (dDPBS). Mean values ± SE are given. $^{*}P < 0.01$, $^{**}P < 0.005$ *versus* control ($n = 5$). B: erythrocyte counts in peripheral blood cells after the administration of PHZ (25 mg/kg, intraperitoneally, arrowhead). Closed circles, treated animals; closed squares, controls. Mean values ± SE are given. $^{*}P < 0.0005$, $^{**}P < 0.0001$ *versus* control ($n = 5$). The statistical significances of the differences were assessed by Student's *t*-test. C–H: peripheral blood cells stained with MGG (C–E) or TB reagents (F–H). C, F: peripheral mature erythrocytes with condensed nuclei. D, G: peripheral blood cells from Day 20 soluble *xl*EPOR-treated *Xenopus*. Immature erythrocytes with less condensed chromatin appeared. E, H: peripheral blood cells from Day 10 of PHZ-treated *Xenopus*. Bars represent 20 μm.

ing erythropoietic activities are conserved. The deduced amino acid sequence of the mature protein was divided into three domains by comparison with the aligned sequences of EPOR molecules (Fig. 2A) and the hydropathy index (Fig. 2B). The extracellular, transmembrane and cytoplasmic domains were predicted to comprise residues Glu^{1}–Arg^{216}, Leu^{217}–Met^{239} and Arg^{240}–Ser^{493}, respectively. In the extracellular domain, cysteine resi-

dues and the WSXWS motif are positioned just outside the transmembrane region, although two additional cysteine residues are present. In the cytoplasmic region, Box 1/2 motifs, which are necessary for intercellular signaling, are conserved (Fig. 2A). No tyrosine kinase catalytic domain exists in the cytoplasmic domain of the molecule, whereas seven tyrosine residues, which are thought to be phosphorylated when the molecule is activated by ligand binding, are present. There is a 55 residue insertion, including one tyrosine residue, after the Box2 motif, and this insertion also exists in the putative *xt*EPOR sequence. When we analyzed the amino acid sequence in this region by BLAST searching in the Ensemble and DDBJ databases, no similar sequence related to mammalian EPORs was obtained.

xlEPOR Are Specifically Expressed in Erythroid Lineage Cells—Northern analysis was performed to confirm *xl*EPOR mRNA expression in various tissues. The *xl*EPOR mRNA was specifically detected in peripheral blood cells. Three mRNA populations of different sizes (5.8, 4.1, 0.6 kb) were detected (Fig. 3A). When *xl*EPOR mRNA from mature and immature erythrocytes were analyzed, the 4.1 and 0.6 kb *xl*EPOR mRNA were more predominantly detected in immature erythrocytes (Fig. 3B). *In situ* hybridization of *xl*EPOR mRNA in peripheral blood cells revealed the *xl*EPOR mRNA to be expressed in immature erythrocytes collected from anemic *X. laevis* induced by PHZ injection (Fig. 4A) and basophilic cells. Immature erythrocytes also express *gata*-1 mRNA (Fig. 4C). We could not detect *xl*EPOR expression in mature erythrocytes or thrombocytes (Fig. 4A). To determine the localization of *xl*EPOR proteins in erythrocytes, immunostaining was conducted using *xl*EPOR PoAb. The *xl*EPOR proteins were detected on the cell surface membranes of immature erythrocytes (Fig. 4E) and basophilic cells in the peripheral blood, while no positive signals were detected in mature erythrocytes or thrombocytes (Fig. 4F). These data indicate that *xl*EPOR localizes on the cell surface membrane, as expected from the sequence motif analysis (Fig. 2B).

Development of Anemia Induced by In Vivo Administration of Soluble xlEPOR—To examine the biological functions of *xl*EPOR *in vivo,* soluble *xl*EPOR containing the extracellular domain of *xl*EPOR was produced in *E.coli*, and purified soluble *xl*EPOR was administrated to male *X. laevis* by intracardiac injection. In contrast to PHZ-injected animals (Fig. 5B), the peripheral erythrocyte number gradually decreased in soluble *xl*EPOR-administrated *Xenopus* (Fig. 5A). Sixteen days after the first injection, the decreased erythrocyte number in soluble *xl*EPOR-injected animals reached a minimum at approximately 60% of control ($761.2 \pm 66.2 \times 10^9$ cells per liter in soluble *xl*EPOR-administrated *Xenopus versus* $1187.6 \pm 54.1 \times 10^9$ cells per liter in controls, P = 0.001). The erythrocyte number recovered from day 16, while the number of leukocytes and thrombocytes did not change significantly. To distinguish between mature and newly produced immature erythrocytes in the peripheral blood, MGG and TB staining were performed. In soluble *xl*EPOR-administrated *Xenopus*, immature erythrocytes, which have less condensed chromatin and basophilic cytoplasm, appeared in the peripheral blood (Fig. 5, D and G), as observed also in the PHZ-injected *Xenopus* (Fig. 5, E and H). Immature erythrocytes were not found in the controls (Fig. 5, C and F). To analyze the ratio of mature and immature erythrocytes in the peripheral blood, the number of immature erythrocytes stained with the TB reagent was determined (Fig. 5G). TB-positive erythrocytes started to increase after 16 days and reached the highest value on day 20 (5.57 ± 0.653% in soluble *xl*EPOR-administrated *Xenopus* versus 0.998 ± 0.329% in control *Xenopus*, $P = 0.0002$) (Fig. 5A).

DISCUSSION

As the result of a genomic duplication event that is estimated to have occurred approximately 30 million years ago, the genome of *X. laevis* is reported to be tetraploid (*15*). Several transcripts in *X. laevis*, including the *gata*-1 used in this study (*14*), have been reported to be derived from different chromosomes (*16*). Therefore, at the beginning of this study, we expected that multiple transcripts of the *xl*EPOR gene might be identified among the isolated three clones. However, the sequence of each clone was identical, and other *xl*EPOR transcripts could not be found. Erythropoiesis is considered to be a fundamental system in vertebrates. Therefore, it might be considered that the EPO and EPOR systems in phyla have structurally and functionally common features; the sequences of the EPO and EPOR molecules, and the immunoreactivity of each molecule with antibodies, are highly conserved among mammals (*7*, *9*). Consequently, we expected that the molecular similarity in EPOs and EPORs would be highly conserved among vertebrates. At first we attempted to identify and clone *Xenopus* EPO cDNA using murine EPO cDNA as a probe. However we failed to obtain any clones (data not shown), in part because of the low similarity of the sequences. This case seems to differ from a c-kit homologue xkl-1 in *X. laevis*, which shares relatively high identity values with humans (73.3%) and mice (73.6%) (*17*). The sequence identities of *xl*EPOR cDNA and deduced amino acid sequence of *xl*EPOR were found to exhibit low identity values compared to that of mammalian and fish EPOR molecules. Based on this result, we speculated that EPO molecules, ligands for EPOR, would be less well conserved among mammals, fishes and amphibians. Supporting this notion, the putative EPO proteins of *Fugu*, zebrafish (Ensemble accession ID: ENSDARG00000030497), and *Tetraodon nigroviridis* (Ensemble accession ID: AY374507) found in the Ensemble genome databases also share low identity values compared to those of mammals (*11*).

While the entire sequence similarity between *xl*EPOR and mammalian EPOR is quite low, the collected evidence indicates that *xl*EPOR is a mammalian EPOR orthologue in *Xenopus*. The results of northern and *in situ* hybridization revealed that *xl*EPOR mRNA is specifically expressed in peripheral blood cells. Three populations of *xl*EPOR mRNAs (5.8, 4.1 and 0.6 kb) were expressed in normal peripheral blood cells, and the 4.1 and 0.6 kb mRNAs are predominantly expressed in immature erythrocytes collected from anemic *Xenopus*. In mammals, erythrocytes and erythroid progenitor cells include three isoforms generated by splicing variants (*18*). The full length form is the most abundant in late stage erythroid progenitors, while the soluble form and

the truncated form are predominantly expressed in early-stage erythroid progenitors. We were not able to characterize the three populations of *xl*EPO mRNA in this study, since we could not clone the other splicing variant. *In situ* hybridization analysis of peripheral blood cells revealed that *xl*EPOR mRNA is specifically expressed in immature erythrocytes, but not in mature erythrocytes. These *xl*EPOR mRNA expressing cells, which abundantly express the *gata*-1 mRNA, exhibit less condensed chromatin and basophilic cytoplasm on MGG and TB staining, indicating that *xl*EPOR expression takes place in an immature erythrocyte–specific manner. In mammals, the EPOR mRNA is expressed in erythrocyte progenitor cells in bone marrow and spleen. In addition, non-hematopoietic cells, including those of the central nervous system, placenta, oviduct, testis and various cancer cells, are also reported to express EPOR (*19–23*). In *X. laevis*, it is of interest that some basophilic cells were found to express *xl*EPOR (data not shown), because it has also been reported that recombinant human EPO binds to peripheral B-cells in rainbow trout, and induces both cell proliferation and Ca^{2+} influx (*24*).

Various soluble cytokine receptors have been shown to arise by proteolytic digestion or alternative splicing, and have neutralizing and agonistic effects toward their receptors (*25*). For example, soluble IL-6 receptor binds to IL-6, and the soluble IL-6R/IL-6 complex stimulates human megakaryocyte progenitors through gp130 signaling *in vivo* (*26*). Soluble c-MPL and soluble G-CSF-R, interacting with their respective cellular receptors, stimulate the proliferation of murine primitive hematopoietic progenitors (*27*). The neutralization of ligand functions has been achieved by using the extracellular domain of the receptor, such as in the case of GST-fusion or Fc fusion proteins (*28*, *29*). We used soluble *xl*EPOR proteins to investigate biological functions in *Xenopus* erythropoiesis, since we were not able to apply an established natural source of *xl*EPOR ligand (EPO). We postulated that *in vivo* administrated soluble *xl*EPOR would bind to endogenous EPO to inhibit EPO-*xl*EPOR binding on target cells, thereby impairing erythropoiesis. In contrast to the hemolytic acute anemia induced by PHZ, the peripheral erythrocyte counts of soluble *xl*EPOR-administrated *X. laevis* gradually decreased. The peripheral erythrocyte counts in the soluble *xl*EPOR-injected animals reached a minimum on day 16 after the first injection (Fig. 5A), while those in the PHZ-injected animals reached the minimum at day 9 (Fig. 5B). These data suggest that the target cells of soluble *xl*EPOR are *xl*EPOR-expressing immature erythrocytes (Fig. 4, A and E), while PHZ induces acute hemolysis in both immature and mature erythrocytes. The peripheral erythrocyte counts of soluble *xl*EPOR-injected animals did not decrease below 60% of controls, and started to recover despite consecutive soluble *xl*EPOR injections. Certain points merit consideration in this regard, as follows: (i) Endogenous EPO production is accelerated in response to the moderate erythrocytopenia induced by soluble *xl*EPOR, and the amount of endogenous EPO exceeds the neutralizing capacity of soluble *xl*EPOR. Supporting this possibility, erythropoiesis occurs anew after the nadir; meanwhile, basophilic immature erythrocytes emerge in the peripheral blood (Fig. 5, D and G). (ii) Antibodies against structurally artificial portions of recombinant soluble *xl*EPOR were produced, and then exogenous soluble *xl*EPOR activity was neutralized *in vivo*. (iii) As TPO enhances erythropoiesis in the developing embryo in *X. laevis* (*10*), other factors act on erythrocyte progenitors to compensate erythropoiesis instead of *Xenopus* EPO.

In conclusion, we have identified a mammalian homologue of EPOR in *X. laevis*. To the best of our knowledge, this is the first report to demonstrate a significant biological role of an EPOR homologue in non-mammalian erythropoiesis. The identification of the ligand for *xl*EPOR should be of importance, since the physiological functions and cellular signaling through *xl*EPOR toward cellular proliferation and differentiation in erythropoiesis is not elucidated at present. The study on *Fugu* EPO, which lacks a flanking hypoxia-responsive element (*11*, *30*), has suggested the possibility of great diversity in the regulation of erythropoiesis among phyla. The new animal model in *X. laevis* developed in this study should prove to have utility in the quest to understand adult hematopoiesis in non-mammalian vertebrates.

The nucleotide sequence for the *Xenopus laevis* EPOR cDNA sequence has been deposited in the GenBank database under Accession number (AB189477). This research was supported in part by a Waseda University Grant for Special Research Projects, and partly by the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for the Bio-venture Project, and for Scientific Research. The authors would like to thank Dr. Takahiro Ochiya (The National Cancer Center Research Institute) for valuable and critical discussions about various aspects of this work.

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