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RESEARCH ARTICLE

Identification of erythroid progenitors induced by erythropoietic activity in *Xenopus laevis*

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SUMMARY

Oxygen is essential for the survival of animals. Red blood cells in the circulation, i.e. peripheral erythrocytes, are responsible for transporting oxygen to tissues. The regulation of erythropoiesis in vertebrates other than mammals is yet to be elucidated. Recently we identified erythropoietin, a primary regulator of erythropoiesis, in *Xenopus laevis*, which should enable us to identify target cells, including erythroid progenitors, and to investigate the production and development of erythroid cells in amphibians. Here, we established a semi-solid colony-forming assay in *Xenopus laevis* to clarify the existence of colony-forming unit-erythroid cells, the functional erythroid progenitors identified *in vitro*. Using this assay, we showed that recombinant *xl*EPO induces erythroid colony formation *in vitro* and detected an increased level of erythropoietin activity in blood serum during acute anemic stress. In addition, our study demonstrated the possible presence of multiple, non-*xl*EPO, factors in anemic serum supportive of erythroid colony formation. These results indicate that erythropoiesis mediated by erythropoietin is present in amphibian species and, furthermore, that the regulatory mechanisms controlling peripheral erythrocyte number may vary among vertebrates.

Key words: EPO, phenylhydrazine, anemia, erythropoiesis, *Xenopus laevis*, colony assay.

INTRODUCTION

Oxygen is required for sustaining the life of animals through the production of energy (Bunn and Poyton, 1996). In vertebrates, erythrocytes carry oxygen to tissues; therefore, the maintenance of peripheral erythrocyte number must be tightly regulated to control oxygen homeostasis. The systems of oxygen intake, transport and consumption vary among animals depending upon their body structure or habitat. For example, fish take up oxygen through gills; amphibians can take up oxygen through both wet skin and lungs; birds use air sacs to help respiration. Because oxygen is vital for all life, yet animals have evolved different mechanisms to keep oxygenated, it is interesting to study in depth how erythropoiesis is regulated in different species.

All circulating blood cells originate from multipotent hematopoietic stem cells capable of self-renewal and differentiation into divergent cell lineages. The principal factor for the regulation of erythropoiesis is erythropoietin (EPO), a hematopoietic factor that binds to its receptor EPOR. At sites of erythropoiesis, EPO stimulates burst-forming unit-erythroid cells (BFU-e) and, subsequently, colony-forming unit-erythroid cells (CFU-e), which are known to express EPOR on the cell surface to promote survival, proliferation, and differentiation towards the erythrocytic hematopoietic lineage (Broudy et al., 1991; Gregory and Eaves, 1977; Gregory and Eaves, 1978; Sawada et al., 1987). In adult humans and rodents, EPO is produced in the kidney and transported to the bone marrow where erythropoiesis occurs (Erslev, 1953; Jacobson et al., 1957; Reissmann et al., 1960). The transcription of EPO mRNA increases 100-fold in response to hypoxia (Blanchard et al., 1993). The path of terminal differentiation from an erythroid progenitor cell goes through pronormoblast, basophilic normoblast, polychromatophilic normoblast, orthochromatophilic normoblast and reticulocyte to produce the mature erythrocyte (Elliott et al., 2008). Enucleation precedes reticulocyte formation in mammals.

CFU-e is defined as an erythropoietic progenitor capable of colony formation in semi-solid culture. This semi-solid culturing is known as a colony assay, which is one of a number of methods used to identify progenitors as well as to quantify hematopoietic factors (Haga and Falkanger, 1979). In response to anemic stress the blood EPO level increases and stimulates erythroid progenitors *in vivo* and thus recovery from anemia. In addition, pre-existing erythroid progenitors, BFU-e, form bursts of erythroid colonies (Gregory and Eaves, 1977; Gregory and Eaves, 1978). Prior to commitment to the erythroid lineage, hematopoietic progenitors such as CFU-EM (CFU-erythrocyte/megakaryocyte), CFU-GEM (CFU-granulocyte/erythrocyte/macrophage), CFU-GEMM (CFU-granulocyte/erythrocyte/monocyte/megakaryocyte) and CFU-Mix (CFU-mixed cells) have the potential to differentiate into more than one lineage of cells in response to various cytokines (Ash et al., 1981). The colony assay is the only way to identify such functional progenitors, retrospectively, *in vitro*.

Detailed information is currently available on the molecular mechanisms of hematopoietic development in rodents and primates; however, this process remains to be elucidated in non-mammalian vertebrates. Recently, erythropoietin genes in fish were identified

and their role in erythropoiesis confirmed (Chou et al., 2004; Chu et al., 2007; Paffett-Lugassy et al., 2007). Comparative studies provide a better understanding of the mechanisms of hematopoiesis and variations in its regulation throughout evolution. Therefore, we investigated hematopoiesis in *Xenopus laevis* (*X. laevis*), the African clawed frog, in order to elucidate the cellular development of hematopoietic cells and their molecular regulation in the amphibian species.

Identification of definitive hematopoietic sites in the amphibian has been hindered by the difficulty in identifying hematopoietic progenitors. On the basis of histological observations, the bone marrow and the spleen have been implicated as the major sites of erythropoiesis in many terrestrial amphibians such as *Rana* (Carver and Meints, 1977; Maslova and Tavrovskaia, 1993). For larval *X. laevis*, the kidney and liver have been considered as possible erythropoietic tissues (Turner, 1988). However, in the adult *X. laevis*, the liver has been suggested as the major site of erythrocyte production following phenylhydrazine (PHZ)-induced anemia (Thomas and Maclean, 1975). It is important to establish a technique to efficiently detect and identify hematopoietic progenitors and hematopoietic regulators in order to further examine the molecular regulation of hematopoiesis. The development of monoclonal antibodies and multi-parameter flow cytometric techniques has led to the successful identification and isolation of hematopoietic progenitor cells in mammals. As no series of surface marker monoclonal antibodies is available for *X. laevis*, in this study we developed a classical method to identify and score hematopoietic progenitor cells. This method, the *in vitro* colony-forming assay, is discussed herein.

In this report, we reveal the presence of CFU-e in the amphibian species *X. laevis*. By establishing a colony assay, we were able not only to demonstrate the presence of erythroid progenitors in the liver capable of forming erythroid colonies *in vitro* but also the specific effect of recombinant *X. laevis* EPO (*xl*EPO) and the presence of *xl*EPO in *X. laevis* serum during PHZ-induced anemia. To our knowledge, this is the first report to describe the establishment of a physiological animal model to investigate the erythroid progenitor CFU-e in amphibian species. Taken together with our previous findings about *xl*EPO expression in the anemic state (Nogawa-Kosaka et al., 2010), we show that the mechanism of regulation of EPO expression may vary among vertebrate species.

MATERIALS AND METHODS

Animals, induction of anemia and preparation of anemic serum

Wild-type African clawed frogs (*X. laevis*, Daudin 1802) were purchased from Aquatic Animal Supply (Misato, Japan) and maintained in a light- (12 h L:12 h D) and temperature-controlled (22°C) conventional culture room. All animal experiments in this study were conducted according to the Regulations for Animal Experimentation of Waseda University, Tokyo, Japan.

Anemia was induced in male *X. laevis* (40 g) by administration of PHZ (Sigma, St Louis, MO, USA) on day 0 as previously described (Chegini et al., 1979). Blood samples were collected from the heart after anesthetizing the frogs in ice-cold water. The blood was allowed to clot for 24 h at 4°C. Samples were then centrifuged at 15,000 ***g*** for 60 min at 4°C to obtain serum. Serum was applied to a NAP-5 column (GE Healthcare, Tokyo, Japan) to remove residual PHZ and the product was concentrated to the initial volume by ultrafiltration (5000 nominal molecular weight limit, NMWL; Millipore, Carrigtwohill, Co. Cork, Ireland). Samples were then sterilized using centrifugal filter units (Millipore) and frozen until use. The frogs were killed before dissection by breaking their spine.

Cell harvesting and blood cell count

To isolate hematopoietic cells, livers were dissected and placed in diluted (0.8×) Dulbecco's modified phosphate-buffered saline (dDPBS) or 0.8× alpha minimum essential medium (α-MEM) (Gibco, Invitrogen, Tokyo, Japan). We used dDPBS(–) (dDPBS without Mg^{++} and Ca^{++} ions) in our experiments in order to avoid blood coagulation. Organs were washed, minced and drawn in and out of a syringe through a 27 gauge needle to make a single-cell suspension, which was filtered through a 40 μm nylon cell strainer (BD Falcon; BD Biosciences, San Jose, CA, USA). Blood cells were stained with Shaw's diluting solution and counted with a hemocytometer (Hadji-Azimi et al., 1987).

Cytology

Cytocentrifuged preparations were made with 2×10^5 cells in dDPBS(–) containing 10% fetal calf serum (FCS) and centrifuged at 1100 ***g*** for 4 min onto glass slides. After air drying, cytocentrifuged preparations were stained with May–Grünwald–Giemsa (MGG) (Wako, Osaka, Japan) and *o*-dianisidine (Wako) following the manufacturer's instructions, and examined by light microscopy (model BX51; Olympus, Tokyo, Japan). Blood cells were observed based on a previous report (Hadji-Azimi et al., 1987).

Histology and immunohistochemistry

Xenopus laevis livers were dissected, fixed overnight in Bouin's solution, embedded in paraffin and sectioned. Sections were stained with hematoxylin and eosin (HE) and *o*-dianisidine solution. For immunohistochemistry, fresh frozen sections of 10 μm thickness were fixed in 4% ice-cold acetone for 10 min. Sections were blocked with 4% BlockAce (Snow Brand, Tokyo, Japan) and washed with Tris-buffered saline (TBS); immunostaining was then performed with 1 μg anti-*xl*EPOR rabbit polyclonal primary antibody (Aizawa et al., 2005) and secondary anti-rabbit IgG conjugated to Alexa488 (Invitrogen, Carlsbad, CA, USA). 4′,6′-Diamidino-2-phenylindole (DAPI) (Sigma) was used to stain the nuclei.

In vitro colony assay

Hematopoietic cells were isolated from organs of *X. laevis* (10–20 g). Cells were washed with 0.8× α-MEM. Semi-solid cultures (0.5 ml) for the erythroid colony-forming activity assay comprised 0.8×10^5 cells $well^{-1}$ unless otherwise stated, supplemented with 100 μg ml^{-1} streptomycin (Invitrogen), 100 μg ml^{-1} kanamycin (Invitrogen), 0.8% methylcellulose (Shin-Etsu Chemical, Tokyo, Japan) and 20% heat-inactivated FCS. Stimulators or antibodies were added where appropriate. Colony-forming assays were performed in triplicate in 24-well cell culture plates (Corning Inc., Corning, NY, USA). After 2 days of incubation in 5% CO_2 at 23°C, erythroid colonies were scored *in situ* using inverted phase-contrast microscopy at a magnification of ×200. In mammals, a CFU-e has been defined as a cell that can form a colony consisting of 16–64 erythroblasts. However, in order not to underestimate the erythroid colony number in our *X. laevis* experiment in the absence of evidence from preceding reports, we scored colonies consisting of at least four erythroblasts. Using a dissecting microscope, colonies were picked up with a micropipette, suspended in dDPBS(–) containing 10% FCS and cytocentrifuged. They were then stained to determine the cellular composition of each colony. Further observations were continued up until day 20 to identify the appearance of any erythroid bursts.

Preparation of anti-*xl*EPO peptide antibody

Polyclonal antibodies were generated in New Zealand white rabbits against the synthetic *xl*EPO peptide *xl*EPO-P: 44–57, NH_2-Thr-Lys-

Leu-Asn-Val-Gly-Glu-Trp-Asn-Lys-Leu-Gln-Thr-Ser with an extra Cys residue at the C-terminus to allow conjugation to keyhole limpet hemocyanin (KLH). This peptide sequence is predicted (MacVector version 8.0, Accelrys Software Inc., San Diego, CA, USA) to be located on the surface of the *xl*EPO because of its higher hydropathy and antigenic index. Elevation of antibody titer was confirmed by enzyme-linked immunosorbent assay (ELISA). The IgG fraction containing antibody to *xl*EPO peptide was prepared by partial purification with a protein G affinity column (Prosep-G; Millipore), and subsequently antibody specific to the antigen (anti-*xl*EPO[44–57] antibody) was purified using antigen peptide immobilized on a Sepharose column. Antibody specificity was determined by western blot and *xl*EPOR-FDCP2 cell proliferation assay. The *xl*EPOR-FDCP2 cell proliferation assay is able to examine the biological activity of recombinant *xl*EPO as performed in our previous study (Nogawa-Kosaka et al., 2010). Recombinant *xl*EPO used in the *xl*EPOR-FDCP2 cell proliferation assay was produced in COS cells. COS-1 cells were provided by the RIKEN BioResourse Center (Ibaraki, Japan) through the MEXT National Bio-Resource Project, Japan.

Fractionation of anemic serum by lectin column chromatography

PHZ day 4 anemic *X. laevis* serum was fractionated by concanavalin A (ConA) Sepharose (Amersham Biosciences, Uppsala, Sweden) followed by wheat germ agglutinin (WGA) agarose (Honen, J-Oil Mills Inc., Tokyo, Japan). Unbound proteins were collected by washing with 20 mmol l^{-1} Tris-HCl (pH 7.4) containing 0.15 mol l^{-1} NaCl (TBS) or 20 mmol l^{-1} Tris-HCl (pH 7.4) containing 0.5 mol l^{-1} NaCl. Bound glycoproteins were eluted with 0.5 mol l^{-1} methyl-α-D-glucoside (α-MG) or 0.5 mol l^{-1} *N*-acetylglucosamine (GlucNAc). The buffer in each fraction was exchanged with DPBS(–) using a NAP-5 column and concentrated to its original volume by ultrafiltration then sterilized as stated above.

Statistics

All cell proliferation assays and colony assay experiments were performed on triplicate samples. Error bars in figures depict standard errors. Differences were accepted as significant at the 95% confidence level ($P<0.05$).

RESULTS

Presence of EPOR-positive cells in the hepatic sinusoid

It is well known that erythroid progenitors exist in the bone marrow of adult mammals. Although the number of erythrocytes is under the control of a homeostatic mechanism, the exact molecular mechanism(s) in erythropoietic organs is unclear in *X. laevis*. In PHZ-induced anemia, the liver is the site that contributes the most to recovery (Chegini et al., 1979), but in the normal adult the site of erythropoiesis has not been confirmed. To determine the sites of erythrocyte production, i.e. the sites at which erythroid progenitors reside, in the normal adult *X. laevis*, the blood cells within various organs were morphologically characterized with reference to a previous report (Hadji-Azimi et al., 1987). The presence of immature hemoglobin-generating erythroblasts, as determined by differential cell counts and identified by *o*-dianisidine staining and MGG staining, indicated that the liver is the predominant site of erythropoiesis in adult *X. laevis* (Table 1). There was no sign of hematopoiesis in the kidney, which serves as an important site of erythropoiesis in fish (Stachura et al., 2009).

Liver sections showed the adult *X. laevis* liver has a similar structure to mammalian liver (Fig. 1A,B), with hepatocytes closely associated with sinusoids in areas where erythrocytes are observed. Examination of liver sections following immunohistochemistry showed that increased numbers of *xl*EPOR-positive cells reside in the hepatic sinusoid of adult *X. laevis* following PHZ-induced anemia (Fig. 1C,D).

Erythroid progenitors, CFU-e, reside in the liver

In order to better examine the production of functional erythroid progenitors with the ability to proliferate and differentiate, we developed, through a trial-and-error process, a semi-solid colony assay and then optimized the procedure (Fig. 2A) by modifying established methods of mammalian colony assay (Metcalf, 1984). When cells taken from adult *X. laevis* liver were cultured in semi-solid medium in the presence of anemic serum, red-tinged compact colonies consisting of more than 16 cells were observed on day 2 of culture that resembled a mammalian erythroid colony (Fig. 2B). The colonies survived for a few days and degenerated on days 4–5. *o*-Dianisidine and MGG staining revealed that the cells constituting the colony were hemoglobin-positive erythroid cells (Fig. 2C). The colony appeared as a heterogeneous erythroid colony comprising mature and immature erythroid cells (Fig. 2C).

Erythropoietic activity in anemic serum is detected by colony assay

The colony count increased in response to an increased concentration of anemic serum (Fig. 3A). Additionally, as the seeding liver cell number was increased, the erythroblast colony formation increased non-linearly (Fig. 3B). There were no CFU-e-derived colonies formed by kidney or bone marrow (data not shown). Those colonies that were formed by spleen cells were red–white heterogenic colonies with a mixture of red-colored cells and white-colored cells (data not shown), indicating that the cells in the spleen are not CFU-e but are more immature cells such as CFU-EM, CFU-GM, CFU-Mix or CFU-GEMM (Y.T., A. Tahara, S. Kinoshita, M.M., T. Ishida-Iwata, M. Obuchi-Shimoji, N.N.-K. and T.K., manuscript in preparation). Together with the observation of erythroblast presence

Table 1. Erythrocyte cell counts in adult *Xenopus laevis* organs

	Liver				Spleen				BM			
	(%)	s.d.	Count	s.d.	(%)	s.d.	Count	s.d.	(%)	s.d.	Count	s.d.
RBC	60.5	8.7	57.4	22.3	13.4	4.3	1.5	0.3	34.5	15.9	0.2	0.1
Eblast	6.7	8.2	5.5	5.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Blast	1.0	0.3	1.0	0.5	2.8	1.2	0.3	0.1	5.1	2.3	0.0	0.0
Others	31.8	4.1	23.4	13.6	83.8	4.6	9.6	1.6	60.3	16.5	0.3	0.2
Total	100.0	21.3	87.4	42.2	100.0	10.1	11.4	2.1	100.0	34.8	0.5	0.4

Data are from 3 individual *Xenopus laevis* (15 g). Differential counts were obtained by identifying at least 500 cells per sample, unless cytocentrifugation resulted in preparations containing fewer cells. Cell count is given $\times 10^6$ cells.
RBC, red blood cell; Eblast, erythroblasts; Blast, hematopoietic precursor cells; BM, bone marrow.

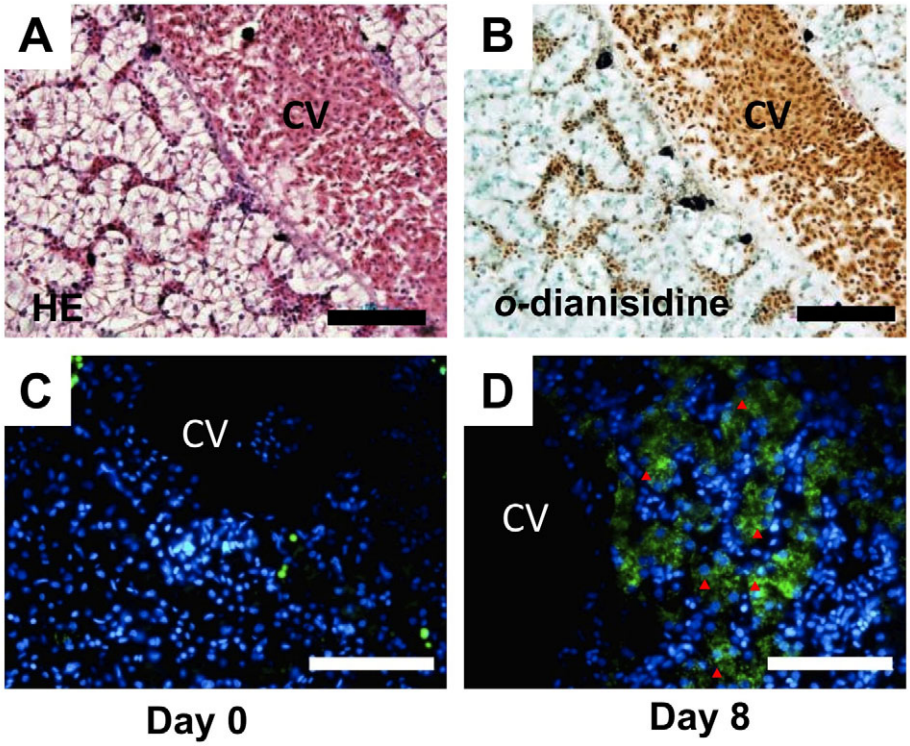

Fig. 1. Erythropoietin receptor (EPOR) expression in the adult liver of *Xenopus laevis*. Hematoxylin and eosin staining (A) and *o*-dianisidine staining (B) of paraffin-embedded sagittal tissue sections (4 μm thickness) fixed in Bouin's solution. CV indicates the central vein. Immunohistochemical staining of normal (Day 0, C) and phenylhydrazine (PHZ)-anemic (Day 8, D) transverse liver sections (10 μm thickness) with *xl*EPOR polyclonal antibody. *xl*EPOR-positive cells reside in the hepatic sinusoid of anemic liver. Arrowheads indicate *xl*EPOR-positive cells, which lie adjacent to the central vein. Nuclei are stained with DAPI (blue). Scale bars represent 100 μm.

in the liver, these data demonstrate that the liver contains cells that are equivalent to the CFU-e found in mice and humans. In contrast, the spleen contains hematopoietic progenitors more primitive than erythroid lineage-committed progenitors. We next examined serial changes in erythropoietic colony-stimulating activity in response to PHZ-induced anemic sera (Fig. 3C). Erythroid progenitors from liver responded to anemic sera to form erythroblast colonies. Day 4 serum stimulated the greatest erythroid colony formation, while day 8 serum stimulated half this number of erythroid colonies, indicating that erythropoiesis activity is controlled by some humoral factor.

Anemic serum contains increased levels of *xl*EPO activity

EPO has been considered as a master regulator of mammalian erythropoiesis. Previously, we identified *xl*EPO and showed its expression profile (Nogawa-Kosaka et al., 2010). As in mammals, *xl*EPO might play a key role as a regulator of erythropoiesis in *X.*

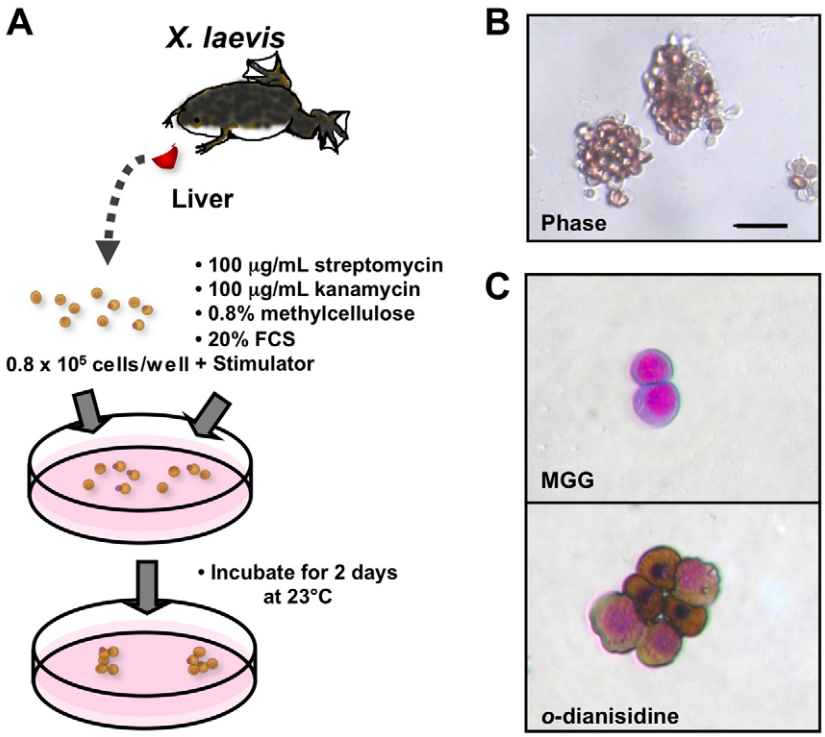

Fig. 2. Identification of hepatic erythroid progenitors in the semi-solid culture system. (A) Schematic diagram of the *in vitro* semi-solid colony formation assay. Stimulators were either recombinant *X. laevis* erythropoietin (*xl*EPO) or *X. laevis* serum. FCS, fetal calf serum. (B) Morphology of hemoglobin-synthesizing colonies derived from *X. laevis* liver cells after 2 days of culture in methylcellulose semi-solid medium in the presence of 1% day 4 anemic serum. Scale bar represents 20 μm. (C) Colony-derived cells cytocentrifuged onto slides and stained with May–Grünwald–Giemsa (MGG) and *o*-dianisidine. These pictures are representative of more than 20 independent experiments. MGG staining revealed that the colony-constituting cells are very immature hematopoietic cells with a large nucleus to cytoplasm ratio (cytoplasm stained blue). *o*-Dianisidine staining revealed that they are hemoglobin-positive erythroblasts.

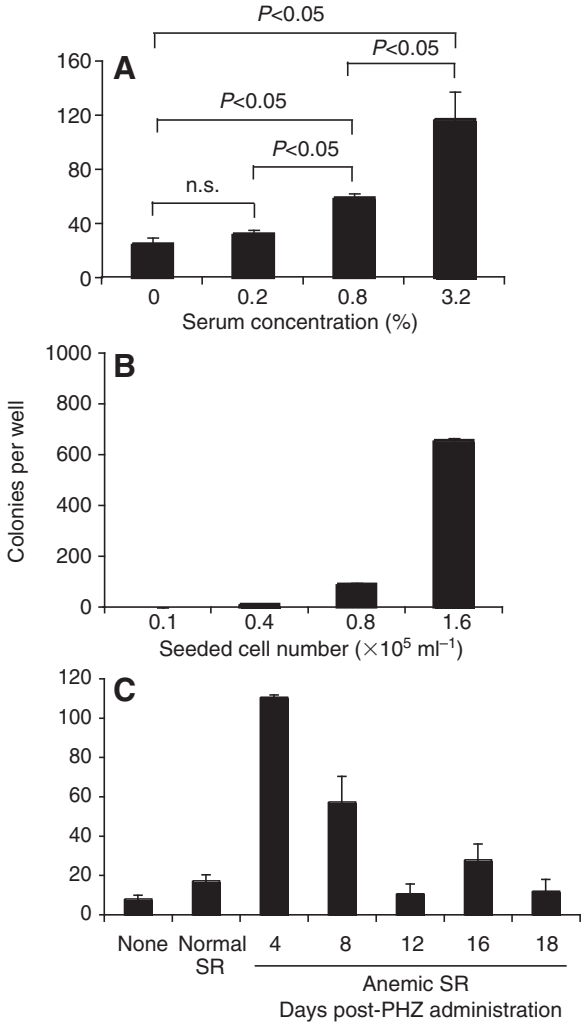

Fig. 3. Colony formation from liver cells in response to erythropoietic activity in anemic serum. Dose–response effects of anemic serum (A) and initial seeding cell concentration (B) on colony formation. Day 8 serum was used as it was the most anemic day in the PHZ-anemic model. (C) Erythroid colony-forming activity of anemic sera. None, no addition; Normal SR, normal serum; Anemic serum, serum (1%) from anemic *X. laevis* taken 4, 8, 12, 16 and 20 days after PHZ injection. Error bars indicate standard errors of triplicate assays. n.s., not significant.

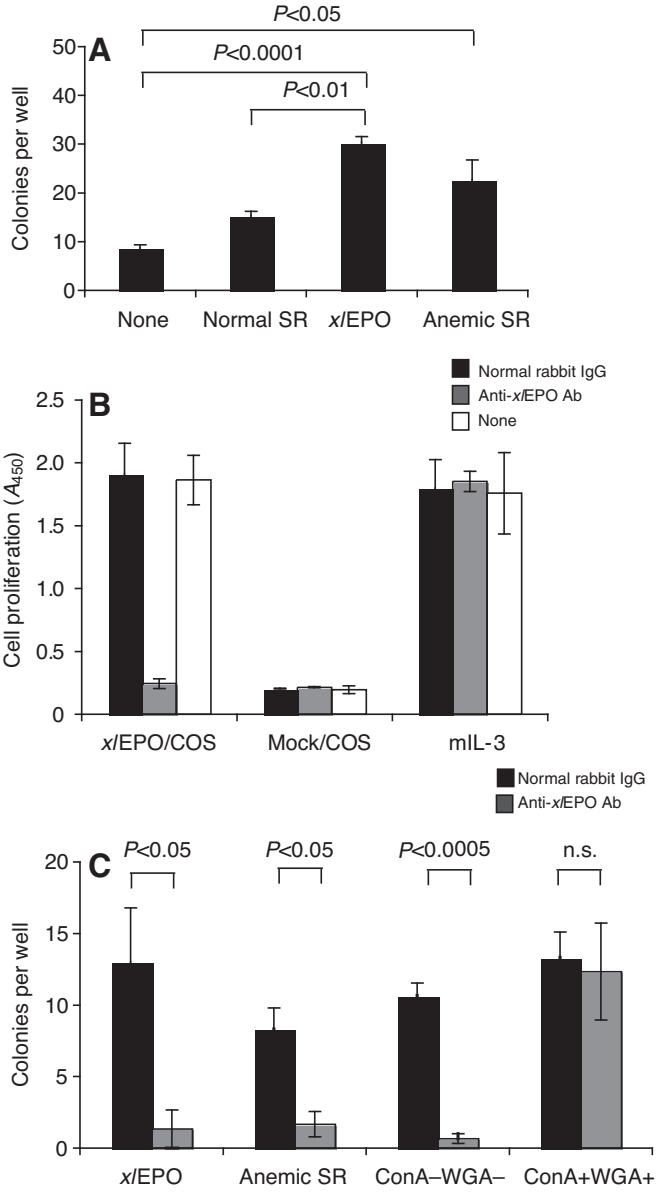

Fig. 4. Comparative characterization of erythroid colony formation induced by recombinant *xl*EPO and native erythropoietic activity in fractionated anemic serum. (A) Colony assay showing that *xl*EPO and anemic serum have erythroid colony-stimulating activity. Normal and anemic day 4 sera were added to wells at a final concentration of 4.4%. Recombinant *xl*EPO (see Results) at a final concentration of 2 ng ml^{-1} was added to the semi-solid culture to assess erythropoietic activity. None, no additions. (B) Anti-*xl*EPO[44–57] antibody inhibits proliferation of *xl*EPOR-FDCP2 cells. *xl*EPO/COS (2% conditioned media), Mock/COS (2% conditioned serum) and interleukin-3 (IL-3, 0.1 ng ml^{-1}) were added to the cell line *xl*EPOR-FDCP2 (see Materials and methods). Normal rabbit IgG or anti-*xl*EPO[44–57] antibody was added to each sample. A_{450}, absorbance at 450 nm. (C) Colony-forming activity of *xl*EPO and anemic serum was inhibited by anti-*xl*EPO[44–57] antibody. Erythropoietic activity of the concanavalin A (ConA)-negative, wheat germ agglutinin (WGA)-negative anemic serum fraction (4.4%) was inhibited by anti-*xl*EPO[44–57] antibody; residual activity of the ConA-positive, WGA-positive fraction was not affected. Normal rabbit IgG (50 μg ml^{-1}) was added as a control. Error bars indicate standard errors of triplicate assays. n.s., not significant.

laevis. Based on this assumption, we produced recombinant *xl*EPO in *E.coli*, purified it to homogeneity (M.M., K.N., M.A., N.N.-K., R.K. and T.K., manuscript in preparation) and used it as a stimulator in the current colony-forming assay. When recombinant *xl*EPO was added to the semi-solid culture, erythroid colony formation significantly increased (Fig. 4A). To find out whether this effect was due to recombinant *xl*EPO activity, we produced anti-*xl*EPO[44–57] antibody and included it in the culture media. This antibody was confirmed to inhibit the proliferation of *xl*EPOR-FDCP2 cells (Fig. 4B). As shown in Fig. 4C, the colony formation by recombinant *xl*EPO was inhibited by addition of this antibody.

From this result, we postulated that native *xl*EPO was contained in the anemic serum that had shown erythropoietic activity in our current colony assay study, and showed that most of the stimulation coming from the anemic serum was attributable to *xl*EPO (Fig. 4C). However, some residual erythropoietic activity was found in the antibody-treated anemic serum (Fig. 4C). We considered that this was due to non-EPO cytokines, which could support erythroid colony formation in the absence of *xl*EPO. In order to confirm this

notion, we fractionated the anemic serum with WGA and ConA lectin column chromatography and investigated the erythropoietic activity by colony assay. Our previous report (Nogawa-Kosaka et al., 2010) showed that *xl*EPO has no glycosylated sites. Therefore the *xl*EPO activity should appear in the ConA-negative WGA-negative fraction – and, indeed, the erythroid colony-forming activity in the ConA-negative WGA-negative fraction was inhibited by the addition of anti-*xl*EPO[44–57] antibody (Fig. 4C). In addition, residual activity was observed in the ConA-positive WGA-positive fraction and was not obscured by the anti-*xl*EPO[44–57] antibody (Fig. 4C). These results indicate that although *xl*EPO is a master regulator of erythropoietic cytokines in anemic serum, there are other, glycosylated, factors in anemic serum that stimulate erythroid colony formation.

DISCUSSION

Following the discovery that colony-forming unit-spleen (CFU-S) are primitive hematopoietic progenitors (Till and McCulloch, 1961), the development of culture systems to detect different types of hematopoietic colonies in semi-solid culture has contributed greatly to our understanding of cellular differentiation and factors regulating lineage specification during hematopoiesis. However, to date, much of the progress in understanding the hematopoietic regulation leading to the production of various blood cells from common progenitors has been limited to mammalian systems.

The salient points of the present study in *X. laevis* are (1) the establishment of a semi-solid culture colony-forming assay, (2) the identification of erythroid progenitors as colony-forming cells, (3) the identification of the adult liver as the predominant erythropoietic organ, (4) the determination of the presence of *xl*EPO activity in anemic serum and (5) the presence of erythropoietic factors other than EPO in anemic serum.

We established a semi-solid culture assay and found erythroid progenitors in the adult *X. laevis* liver. Anemic serum collected from PHZ-induced anemic *X. laevis* stimulated erythroid colonies from liver cells. The morphology of the typical frog erythroid colony resembled a mammalian erythroid colony (Fig. 2B). The seeded cell numbers and colony numbers were not related linearly (Fig. 3B), probably due to unidentified factor(s) released in the culture that stimulate erythroid colony formation synergistically. In our previous study, *xl*EPO mRNA was expressed in hepatocytes (Nogawa-Kosaka et al., 2010). Therefore, it is possible that the coexisting hepatocytes contributed to erythroid colony formation *in vitro*. In this study, we used anti-*xl*EPO[44–57] antibody to investigate the colony-stimulating activity of recombinant *xl*EPO and anemic serum. For accurate quantification of the stimulating factor(s), it was necessary to improve the culture system. We did not observe any erythroid burst in our colony assay although the various culture conditions under increasing concentrations of stimulating factor(s) or longer culture periods were examined. This indicates that the cytokine(s) promoting burst formation is absent in this culture.

Our recent work with *xl*EPO showed that the molecule does not possess *N*- or *O*-glycosylated sites (Nogawa-Kosaka et al., 2010). Thus, the activity seen in the ConA,WGA-unbound fractions is probably due to native *xl*EPO; and in the ConA,WGA-bound fraction due to an erythropoietic factor(s) other than EPO. As in mammalian hematopoiesis, it is possible that early acting hematopoietic cytokines or non-lineage-specific factors such as interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) co-stimulate erythroid colony formation *in vitro*. In *X. laevis*, the fractionated serum showed slightly higher erythropoietic activity than the added anemic serum, suggesting that multiple factors support erythroid colony formation synergistically or additively, or that some inhibitors are present in the crude sample of the serum. The erythropoietic activity observed in anemic serum may be attributable to early acting cytokines such as IL-3, GM-CSF and stem cell factor (SCF) among others. To date, it has proved to be extremely difficult to identify hematopoietic factors directly from blood; despite the available knowledge and tools, there are only a few reports of the identification and purification of such humoral factors directly from mammalian blood (Kato et al., 1998).

In our *in vitro* colony-forming culture under current conditions, we detected erythroid progenitors from the liver. However, it is possible to form different types of colony if culture conditions are varied. It is therefore possible to search for other cytokines or to detect hematopoietic progenitors of various lineages. In the present culture, the target cells were not a pure population and co-factors may have affected the culture. We should therefore be able to improve the culture system for further study of hematopoiesis in *X. laevis*.

The detection not only of *xl*EPO activity by colony assay but also of native *xl*EPO in serum of *X. laevis* by western blotting would be required in order to identify the molecular characteristics of *xl*EPO. However, the serum *xl*EPO level is expected to be very low based on the mammalian data (human EPO, 130–230 pg ml^{-1}) (Choi et al., 1996); the procedure is also very difficult to perform and could not be carried out by the present methodology. The detection of native serum cytokine, i.e. thrombopoietin (TPO), *in vivo* using antibody has been carried out in mammals by means of partial purification (Matsumoto et al., 1999), and this is also going to be required for *X. laevis* in order to broaden our understanding of the characteristics and regulation of native *xl*EPO.

In our previous study we reported that *xl*EPO mRNA levels did not significantly increase in response to PHZ-induced anemia (Nogawa-Kosaka et al., 2010). However, the increase in erythropoietic activity, which we attribute to *xl*EPO activity, implies that *xl*EPO protein levels are elevated in the anemic serum. If total *xl*EPO mRNA expression levels remain constitutive under anemic conditions, then the regulatory system may differ from that of humans and rodents: an organism's environment or oxygen consumption behavior varies from species to species. Although the presence of the EPO molecule during erythropoiesis is common among vertebrates, the mechanism for maintaining its oxygen homeostasis may be variable. Clarifying the erythropoietic regulation in non-mammalian species will lead to a broader understanding of how different organisms regulate oxygen homeostasis in response to diverse environmental stimuli.

Based on our current findings we propose that the liver is the major erythropoietic organ in adult *X. laevis*. The liver is the primary EPO-producing organ in fetal mice, and erythropoiesis also takes place predominantly in the fetal liver prior to adult hematopoiesis in the bone marrow. In EPO- or EPO receptor-deficient embryos, definitive erythropoiesis is completely blocked (Wu et al., 1995), meaning that EPO is required for erythrocyte production in fetal hepatic erythropoiesis. In both mice and *X. laevis*, hepatocytes express EPO mRNA. Hepatic erythrocyte production in fetal mice and *X. laevis* appears to be similar, implying that hepatic EPO acts in a paracrine manner.

Under the evolutionary processes classified into phylogeny, ontogeny and habitat preference, medullary hematopoiesis has appeared among terrestrial amphibians (Turner, 1988). However, the hematopoietic organs of aquatic vertebrates, particularly amphibians, have still not been defined in depth. Identification and

application of amphibian hematopoietic cytokines such as EPO, granulocyte colony-stimulating factor (G-CSF) and GM-CSF will enable us to analyze in detail the mechanisms of hematopoiesis, the hematopoietic lineage and its regulation in *X. laevis*. In this regard, the *in vitro* colony-forming assay system described here will facilitate the measurement of hematopoietic activity of molecules in non-mammalian vertebrates in future studies, resulting in an improved understanding of the general principles of hematopoiesis through comparative hematology.

LIST OF SYMBOLS AND ABBREVIATIONS

BFU-e	burst-forming unit-erythroid cells
CFU-e	colony-forming unit-erythroid cells
CFU-EM	colony-forming unit-erythrocyte/megakaryocyte
CFU-GEM	colony-forming unit-granulocyte/erythrocyte/macrophage
CFU-GEMM	colony-forming unit-granulocyte/erythrocyte/monocyte/megakaryocyte
CFU-Mix	colony-forming unit-mixed cells
CFU-S	colony-forming unit spleen
ConA	concanavalin A
DAPI	4′,6′-diamidino-2-phenylindole
dDPBS(–)	diluted Dulbecco's modified phosphate-buffered saline (dDPBS without Mg^{++} and Ca^{++} ions)
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
EPOR	EPO receptor
FCS	fetal calf serum
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HE	hematoxylin and eosin
IGF2	insulin-like growth factor 2
IL-3	interleukin-3
KLH	keyhole limpet hemocyanin
MGG	May-Grünwald-Giemsa
PHZ	phenylhydrazine
TBS	Tris-buffered saline
WGA	wheat germ agglutinin
*xl*EPO	*Xenopus laevis* erythropoietin
α-MEM	alpha minimum essential medium

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