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RESEARCH ARTICLE

Hepatic confinement of newly produced erythrocytes caused by low-temperature exposure in *Xenopus laevis*

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SUMMARY

Diminished erythrocyte count and erythropoiesis have been reported during hypothermia in some ectothermic animals. In this study, the African clawed frog, *Xenopus laevis*, was used to investigate the cause of hypothermia-induced anemia. We developed a new model of hypothermia at 5°C and monitored blood cell count and erythropoiesis on several days. Erythrocyte count declined by 30% on the first day following cold exposure (5°C) and mRNA expression of hemeoxygenase-1 was enhanced 10-fold; accumulation of iron as a result of heme degradation was observed in the liver. One day after low-temperature exposure, erythropoietin mRNA expression was elevated in the liver and lung compared with that at normal temperature (22°C) by qRT-PCR analysis. Examination of liver sections (i.e. the erythropoietic organ) showed an increase in *o*-dianisidine-positive erythrocytes in the hepatic sinusoid 5 days after the onset of low-temperature exposure compared with normal liver. Peripheral erythrocyte count remained low, indicating that newly produced erythrocytes did not migrate from the liver to the circulation during hypothermia. In conclusion, this study reveals hypothermic anemia as being associated with hepatic erythrocyte destruction; prolonged anemia during low-temperature exposure is concomitant with newly produced erythrocytes being confined to the liver and may lead to new insights into vertebrate hematopoiesis.

Key words: low temperature, hypothermia, anemia, erythropoiesis, *Xenopus*.

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INTRODUCTION

Temperature is one of the most important environmental factors affecting the physiology of organisms. Endothermic animals have mechanisms to keep body temperature constant in spite of fluctuations in environmental temperature (Silva, 2006). Ectothermic animals, however, generally do not have a thermoregulatory mechanism, and a decrease in body temperature occurs passively during exposure to low environmental temperature (Salt, 1949).

It has been reported that erythropoietic systems respond to low environmental temperature. Cline and Waldmann demonstrated that leopard frogs (*Rana pipiens*) exposed to low environmental temperature became anemic and suggested it was mediated by down-regulation of erythropoiesis (Cline and Waldmann, 1962). Subsequently, there have been several reports of anemia induced by low-temperature exposure of ectothermic animals. In overwintering European frogs (*Rana esculenta*), erythrocyte count and hemoglobin levels decrease during winter compared with pre-winter values (Sinha, 1983); and in zebrafish (*Danio rerio*), erythropoiesis is down-regulated during low-temperature exposure (Kulkeaw et al., 2010). Hematopoietic responses to environmental temperature have also been reported in endothermic animals. In rats (*Rattus norvegicus*) acclimated to 5°C, an increase in the number of erythrocytes and a decrease in leukocytes and platelets was observed (Deveci et al., 2001). It is thought that an increased erythrocyte count reflects increases in metabolic rate required during low-temperature acclimation in endothermic animals, while in ectothermic animals a lower erythrocyte count reflects decreases in metabolic rate during low-temperature exposure. Although these reports suggest that erythropoietic systems play an important role in adaptation to low environmental temperature in all animals, the mechanism remains unresolved.

Hypothermia is known to affect hematopoiesis. Cases of severe anemia or pancytopenia in hypothermic patients have been reported (Sadikali and Owor, 1974; O'Brien et al., 1982; Daly and Rosenfarb, 1991; Lo et al., 2002; Collins and Danzl, 2006). Establishing the relationship between the causes of anemia and erythropoiesis under hypothermic conditions has been hampered by practical difficulties in inducting low body temperature in small mammals for several hours through lowering of ambient temperature or administration of chemicals with regard to the animals' delicate thermoregulatory mechanism. Thus, a stable and convenient model would aid investigations into hypothermic physiology.

The African clawed frog, *Xenopus laevis*, is an aquatic amphibian whose habitat range covers sub-tropical regions of southern Africa. In nature, *X. laevis* experiences a wide range of seasonal temperatures, and could be exposed to environmental temperatures below 10°C. Moreover, *X. laevis* is ectothermic and therefore, unlike endothermic animals, shows decreases body temperature in response to decreases in environmental temperature. Therefore, cold-acclimated *X. laevis* would be a convenient hypothermic model. Additionally, there is growing knowledge of the hematology of *X. laevis.* We previously identified erythropoietin (EPO), a principal

regulator of erythropoiesis, and its receptor (EPOR) in *X. laevis* (Nogawa-Kosaka et al., 2010; Aizawa et al., 2005). EPOR is expressed in erythrocyte progenitors, and EPOR-expressing cells are localized in the hepatic sinusoid (Aizawa et al., 2005; Nogawa-Kosaka et al., 2011). We hypothesized that hypothermic anemia in *X. laevis* was caused by the down-regulation of hepatic erythropoiesis, and that it was associated with decreased EPO expression, based on previous reports in leopard frogs and zebrafish (Cline and Waldmann, 1962; Kulkeaw et al., 2010). In the current study, we induced anemia by low-temperature exposure in *X. laevis* and investigated the effects of hypothermic conditions on hepatic erythropoiesis.

MATERIALS AND METHODS

Animals

Wild-type male African clawed frogs, *X. laevis* Daudin 1802 (mass 30–40 g), were purchased from Aquatic Animal Supply (Misato, Saitama, Japan). Frogs were housed in plastic tanks at normal room temperature (22°C) with constantly running water before experiments began. For low-temperature studies, plastic tanks containing frogs (1 l of water per frog) were maintained in an incubator (Bio Multi incubator, NK system, Osaka, Japan) at 10 or 5°C. To induce anemia, phenylhydrazine (PHZ; Sigma, St Louis, MO, USA), diluted in 0.8× Dulbecco's modified phosphate-buffered saline (dDPBS), was injected intraperitoneally (25 mg kg^{-1} body mass). Seven days after PHZ injection, frogs were transferred to plastic tanks containing water and maintained in the incubator at 5°C. To label replicating erythrocyte progenitors *in vivo*, animals were intraperitoneally injected with 30 mg ml^{-1} bromodeoxyuridine (BrdU; WAKO, Osaka, Japan) diluted in dDPBS (150 mg kg^{-1} body mass). All experiments were conducted according to the Regulations for Animal Experimentation at Waseda University.

Blood cell analysis

Peripheral blood samples were successively obtained by cardiac puncture with a 27-gauge needle attached to a capillary tube coated with the anti-coagulant EDTA-2Na. Peripheral blood counts were made using a hemocytometer after diluting blood 1:100 in Shaw's diluting solution (Hadji-Azimi et al., 1987). Hemoglobin values were measured using an automated blood cell counter according to the manufacturer's protocol (Sysmex, F-820, Kobe, Japan). Hematocrit values were measured by centrifugation in capillary tubes. Cytocentrifuge preparations were made with 2×10^5 cells in dDPBS containing 10% fetal calf serum (Sigma) and centrifuged at 1100 ***g*** for 4 min onto glass slides. The preparations were stained with May–Grünwald–Giemsa (MGG; Wako) and *o*-dianisidine (Wako), and examined by light microscopy (model BX51; Olympus, Tokyo, Japan). For immunocytochemistry, the cytocentrifuge preparations were fixed with formalin–acetone solution (0.5 mol l^{-1} phosphate buffer, 10% formalin and 60% acetone) for 30 s.

Lifespan of erythrocytes

Circulating erythrocytes were labeled *in vivo* with *N*-hydroxysulfosuccinimide esters of biotin for labeling cell surface proteins (EZ-Link Sulfo-NHS-LC-Biotin; Pierce, Rockford, IL, USA). Activated biotin solution (100 μl) in dDPBS (5 mg ml^{-1}) was injected *via* the heart four times (total 2 mg each). Collected blood was washed with dDPBS containing 2% fetal calf serum and 2 mmol l^{-1} EDTA, and incubated with streptavidin–APC conjugates (BD Pharmingen, San Diego, CA, USA). The samples were analyzed in a flow cytometer (FC500-MPL; Beckman Coulter, Tokyo, Japan) to measure the percentage of biotin-labeled cells. The half-life of erythrocytes was calculated using the following equations:

$$y = a \times e^{f} \quad (1)$$

and:

$$T_{1/2} = -\ln 2 / f\,. \quad (2)$$

The exponential trend line was inserted into the raw data *x* (days)/*y* (% of labeled cells), and Eqn 1 was calculated using MS Excel software (Microsoft Corporation). The factor *f* appearing as the exponent of the equations was used to calculate the half-life ($T_{1/2}$) by Eqn 2. Erythrocyte lifespan was also calculated from the regression line.

Quantitative real-time RT-PCR (qRT-PCR) analysis

qRT-PCR analysis was performed as previously described (Nogawa-Kosaka et al., 2010). Primers against the ribosomal protein 13A gene were used as an internal control. Sequences of primers used for the real-time RT-PCR study are given in Table 1. Target amounts were normalized to levels of the *rpl13a* gene. The mean threshold cycle was used to determine relative expression.

Immunohistology and immunocytochemistry

Frogs were quickly killed by beheading, and the liver and spleen were collected. Tissue pieces were soaked immediately in 10% formalin, fixed for 24 h at 4°C, dehydrated through a graded series of ethanol, embedded in Paraplast (Kendall-Tyco Healthcare, Mansfield, MA, USA) and sectioned at 6 μm at room temperature. The sections were stained with *o*-dianisidine and Hematoxylin–Eosin or a mixture of equal parts 5% potassium ferrocyanide and 5% hydrochloric acid for Perls Prussian Blue staining.

For BrdU detection, paraffin sections were heated in a microwave in 0.01 mol l^{-1} citric acid buffer (pH 6.0) to denature nucleic acids. Non-specific antibody binding was blocked by incubating the slides in 5% normal goat serum (NGS) in 0.8× dTBS (15 mmol l^{-1} Tris-HCl, 0.15 mol l^{-1} NaCl, pH 7.5) for 60 min at room temperature. The sections were incubated with mouse anti-BrdU primary antibody (Dako, Carpinteria, CA, USA) in dTBS containing 0.5% NGS overnight at 4°C. After each antibody incubation, sections were washed three times with dTBS for 5 min. The sections were then incubated for 90 min with biotinylated goat anti-mouse IgG secondary antibody (Invitrogen, Carlsbad, CA, USA) in dTBS containing 0.5% NGS. Finally, slides were incubated for 60 min with Alexa Fluor 488-conjugated streptavidin (Invitrogen) or horse-radish peroxidase-conjugated streptavidin (BioRad, Hercules, CA, USA) and color was developed with diaminobenzidine for 5–10 min. Vinculin (mouse anti-human vinculin-1 antibody, Sigma) detection

Table 1. Primers used for quantitative real-time RT-PCR

Gene	Primer	Sequence (5′–3′)
Ribosomal protein 13A	Forward	GGCAACTTCTACCGCAACAA
	Reverse	GTCATAGGGAGGTGGGATTCC
Hemeoxygenase-1	Forward	AGGATATTGGAAGAGGCCAAAAC
	Reverse	TCTTGGGTCCTCTGCTTCGT
Biliverdin reductase A	Forward	TGAAGAAGGAAGTACAGGGCAAA
	Reverse	AATGCCACTAAACGATGGGAAT
Erythropoietin	Forward	GCACCATCCTCTTCACTTCAC
	Reverse	CTTCACACTAACGAGCAACATTG
Erythropoietin receptor	Forward	GCTGCACTTCCACAATCTTTC
	Reverse	CACTCTGTTGTTTGCCTTTACTG
gata-1a	Forward	CCAAAGAAACGCCTGATTGT
	Reverse	TCTCCACTTGCATTCCGTC

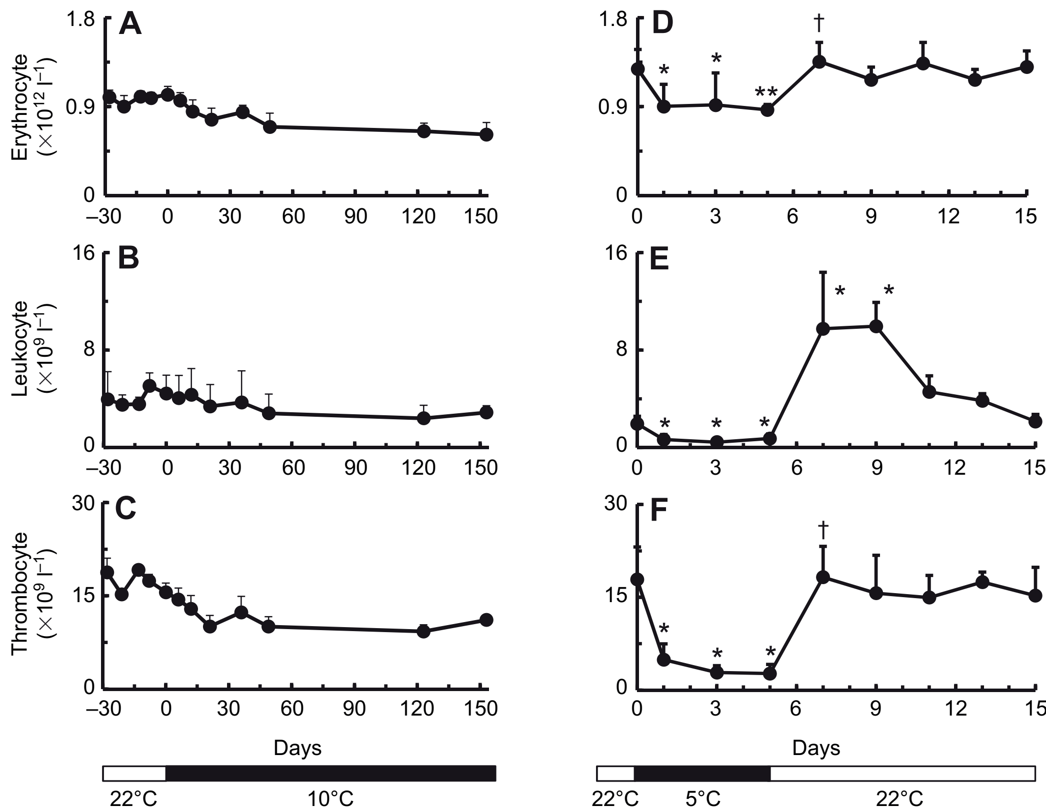

Fig. 1. Peripheral blood count (erythrocyte, leukocyte and thrombocyte) during low-temperature exposure. (A–C) Frogs were exposed to 10°C for 153 days (N=7). (D–F) Frogs were exposed to 5°C for 5 days, and then returned to 22°C for 5–15 days (N=5). *P<0.05, **P<0.01 compared with day 0; †P<0.01 compared with day 5.

was performed as above, using anti-mouse IgG antibody conjugated to Alexa Fluor 568 (Invitrogen).

Statistical analysis

Error bars represent s.e.m. in all figures. Multiple group comparisons were performed by one-way analysis of variance followed by the Tukey–Kramer procedure for comparison of means. Comparisons between two groups were performed using Student's t-test. Values of P<0.05 were considered statistically significant.

RESULTS

Anemia is induced in *X. laevis* by low environmental temperature

Frogs were exposed to a 10°C environment for 153 days (Fig. 1A–C). Erythrocyte count gradually decreased to about 70% of original values by day 49 and thereafter remained low. Leukocytes and thrombocytes followed a similar pattern, decreasing to 83.5% and 60% of original levels, respectively. In non-optimized experiments, many frogs died during low-temperature exposure. In frogs under low-temperature conditions, hemostatic ability and immunity could be low compared with normal frogs, because frogs became chronically leukocytopenic and thrombocytopenic. Thus, sequential blood sampling by consecutive needle puncture could be one of the causes of death. In this study, we established a short-term model exposing frogs to 5°C for 5 consecutive days, and then frogs were put back to 22°C immediately afterwards (Fig. 1D–F). In this model, we focused on investigating the initial stage of erythrocytopenia.

One day after exposure at 5°C, erythrocyte count decreased by approximately 30% and then remained at this level for 5 days (Fig. 1D). Two days after the return to 22°C, erythrocyte count had recovered to initial levels. We compared peripheral erythrocyte parameters from each group at days 0, 5 and 7 (Table 2). Hematocrit and hemoglobin concentrations were significantly reduced on day 5 (Table 2). Recovery to normal levels was observed for these two parameters 2 days after the temperature was restored to 22°C. There were no statistically significant differences in mean corpuscular erythrocyte volume, mean corpuscular hemoglobin content and mean corpuscular hemoglobin concentration between 22°C and 5°C.

Leukocyte count decreased to approximately 40% on day 3, but exceeded the original levels (1.7- and 2.3-fold, respectively) on days 7 and 9 of recovery (Fig. 1E), and gradually settled to initial levels on day 15. The nadir in thrombocyte count was observed on day 3 (approximately 20% of the initial value, Fig. 1F). After the return to 22°C, thrombocyte count recovered to the initial levels by day 7. These results indicate that blood cell count decreased and remained low in response to environmental temperature.

Table 2. Hematologic parameters at low temperature

Groups	RBC (×10^{12} l^{-1})	Hb (g dl^{-1})	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g dl^{-1})
Day 0	0.96±0.08	15.2±1.7	22.0±4.1	231.0±48.7	159.6±0.08	70.9±14.9
Day 5	0.46±0.09**	7.0±2.6*	14.9±2.3*	333.1±93.3	150.4±36.9	49.1±22.0
Day 7	0.87±0.24†	11.1±3.2	18.9±6.0	222.1±57.3	127.8±0.08	63.1±27.6

RBC, erythrocytes; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular erythrocyte cell volume; MCH, mean corpuscular hemoglobin content; MCHC, mean corpuscular hemoglobin concentration.
*P<0.05, **P<0.01 compared with day 0. †P<0.05 compared with day 5.

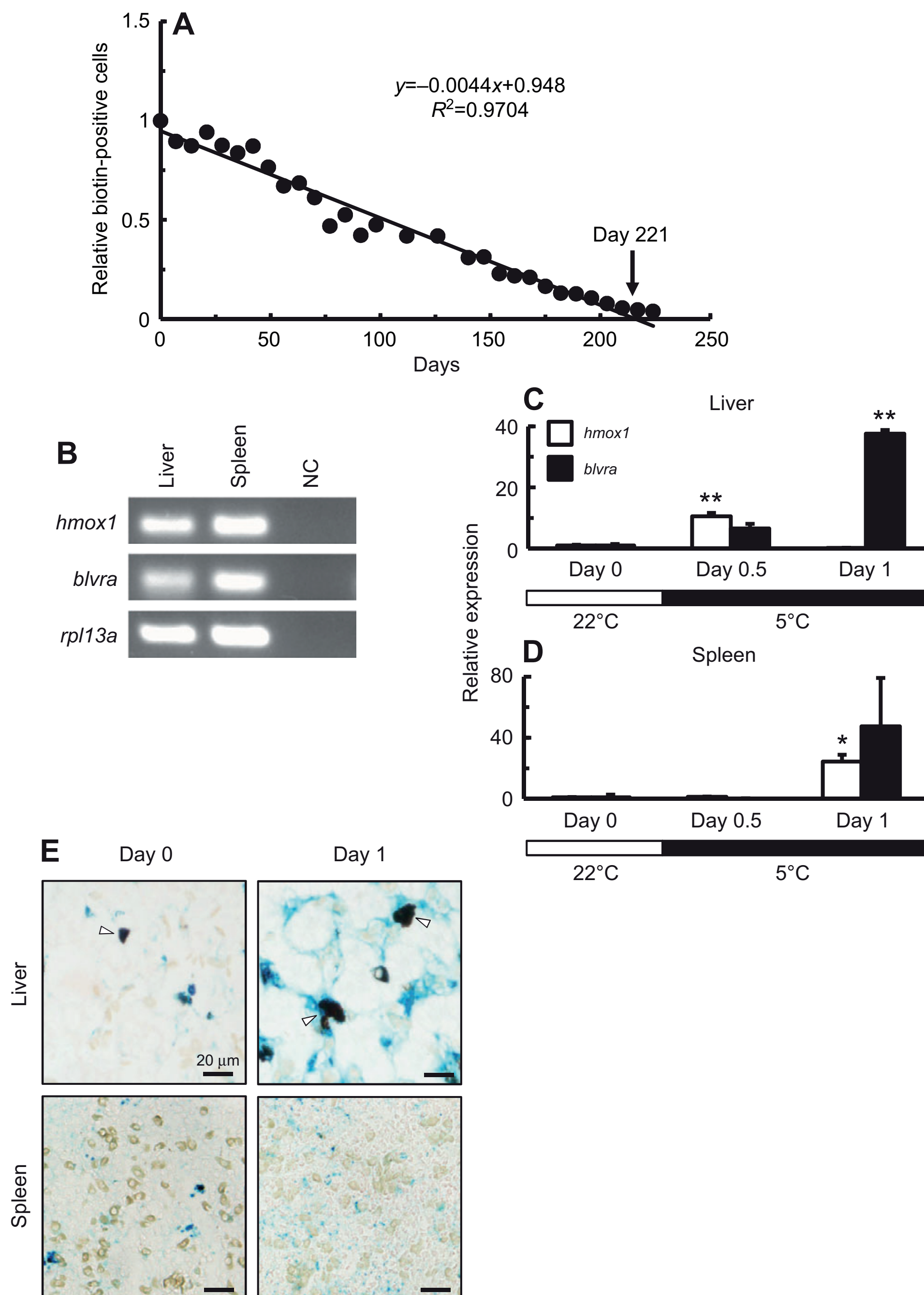

Fig. 2. Analysis of erythrocyte degradation in liver and spleen after exposure to low temperature. (A) Analysis of erythrocyte lifespan in *X. laevis* as the proportion of biotin-labeled cells. (B) mRNA expression of hemeoxygenase-1 (*hmox1*) and biliverdin reductase A (*blvra*) in liver and spleen by RT-PCR. NC, negative control. A housekeeping gene (*rpl13a*) was used as an internal standard. (C,D) *hmox1* and *blvra* mRNA levels in liver and spleen were determined at 0 days (22°C), and 0.5 and 1 day after exposure to 5°C. The data represent relative expression compared with the day 0 group (N=3; *P<0.05, **P<0.001 compared with day 0). (E) Perls Prussian blue-staining of liver and spleen for iron content at day 0, and 1 day after exposure to low temperature. Arrowheads indicate pigmented cells.

Erythrocyte count decreases immediately after exposure to low temperature

First, we attempted to measure erythrocyte lifespan *in vivo* using 5- or 6-*N*-succinimidyloxycarbonyl-fluorescein 3′,6′-diacetate (CFSE) labeling; however, it proved difficult to distinguish CFSE-positive cells from negative cells by 70 days (data not shown). Therefore, instead we used a biotin-labeling method and estimated erythrocyte lifespan to be 220±3.7 days with a half-life of 53.4±5.4 days (Fig. 2C and E). Hence, erythrocyte lifespan in *X. laevis* is longer than in mammals [120 days for humans (Finch et al., 1949); 40 days for mouse (*Mus musculus*) (Van Putten, 1958)] while comparable with that in leopard frog [200 days (Cline and Waldmann, 1962)] and ginbuna carp [*Carassius auratus langsdorfii*, 270 days (Fischer et al., 1998)]. The rate of destruction of erythrocytes in adult *X. laevis* was estimated to be about 0.45% per day under normal conditions from our data of erythrocyte lifespan. Peripheral erythrocyte count decreased by 30% within 1 day of exposure to low temperature (Fig. 1D). It was suggested that enhancement of erythrocyte degradation caused erythrocytopenia after low-temperature exposure. In the normal state, damaged or senescent erythrocytes are degraded by phagocytes in the liver and spleen (Bosman et al., 2005). Liberated heme from hemoglobin is converted to biliverdin, carbon monoxide and free iron by hemeoxygenase, and in turn biliverdin is reduced by biliverdin reductase to bilirubin. In adult *X. laevis*, histological observation indicated that liver and spleen are also the sites of phagocytosis and destruction of damaged erythrocytes (Chegini, et al., 1979). We detected the expression of hemeoxygenase-1 (*hmox1*) and biliverdin

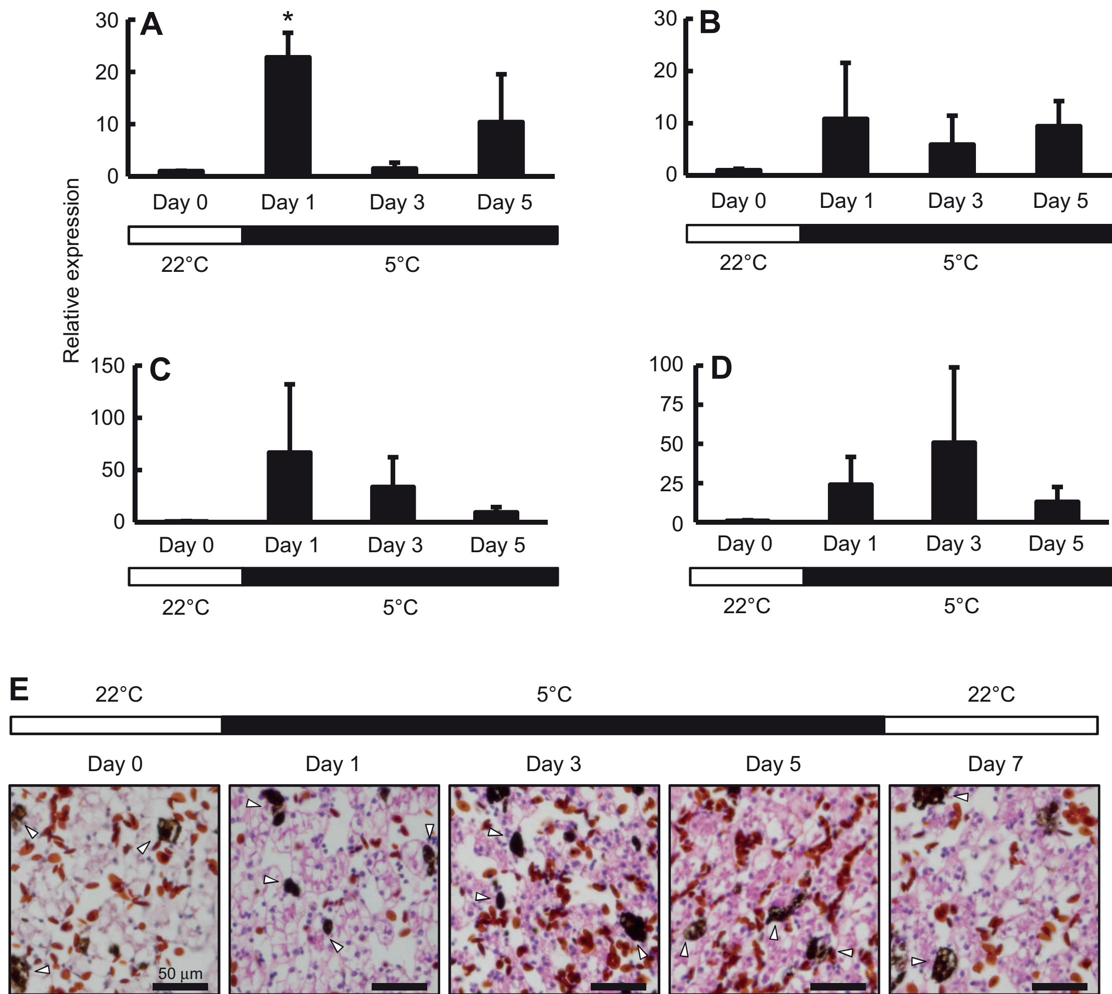

Fig. 3. Erythropoiesis during exposure to low temperature. (A,B) Erythropoietin (*epo*) mRNA levels were determined in lung (A) and liver (B) (*N*=3, $*P<0.05$ compared with day 0). (C,D) Erythropoietin receptor (*epor*, C) and *gata-1a* (D) mRNA expression in erythrocyte progenitors in liver increased after exposure to low temperature (*N*=3). These data represent the relative expression compared with the day 0 group. (E) Distribution of mature erythrocytes in liver after exposure to low temperature. Liver sections were stained with *o*-dianisidine (hemoglobinized erythrocytes stained brown/orange) and Hematoxylin–Eosin. Arrowheads indicate pigmented cells.

reductase A (*blvra*) mRNA in normal liver and spleen (Fig. 2B), which indicates that the site of erythrocyte destruction is liver and spleen in adult *X. laevis*. The expression levels of *hmox1* and *blvra* mRNA in the spleen were higher (3.9-fold and 27-fold, respectively) than in the liver by qRT-PCR (*N*=3). In the liver, *hmox1* increased 10.6-fold, 0.5 days after exposure to low temperature and then declined to normal values after 1 day (Fig. 2C). The expression of *blvra* was elevated 37.6-fold after 1 day at low temperature (Fig. 2C). In the spleen, *hmox1* significantly increased on day 1 (Fig. 2D). *blvra* expression in the spleen showed increased tendency without significance on day 1. Examination of liver sections following Perls' Prussian blue staining showed accumulation of iron on 1 day after low-temperature exposure (Fig. 2E). In the spleen, no significant iron accumulation was observed (Fig. 2E). These results indicate that destruction of erythrocytes in the liver occurs immediately after low-temperature exposure.

Erythropoiesis increases after exposure to low temperature

Next, we analyzed mRNA expression of erythropoiesis-related genes by qRT-PCR during the low-temperature period. In lung, the primary EPO-producing organ in *X. laevis*, *epo* mRNA levels were significantly up-regulated 22.8-fold on day 1 after exposure to low temperature (Fig. 3A). In the liver, *epo* mRNA levels were elevated 10.9-fold compared with day 0 (Fig. 3B). *epor* increased over 50-fold on day 1 and then dropped (33-fold) on day 2 and (10-fold) day 5, i.e. a steady return towards original values (Fig. 3C). In contrast, gata-1a expression appeared to peak later, on day 3 (50-fold), and then declined on day 5 (Fig. 3D). We observed the distribution of mature erythrocytes in liver sections (Fig. 3E). At day 1, the number of *o*-dianisidine-positive mature erythrocytes in the hepatic sinusoid was decreased compared with that on day 0 (Fig. 3E), correlating with enhanced destruction of erythrocytes in the liver (Fig. 2A). Mature erythrocyte numbers increased in the hepatic sinusoid on days 3 and 5 (Fig. 3E). With the return to 22°C, the number of erythrocytes within the liver decreased (day 7). These data indicated that hepatic erythropoiesis is activated in response to acute anemia induced by low-temperature exposure.

Newly produced erythrocytes are confined to liver during low-temperature anemia

To further investigate the effect of low-temperature exposure on erythropoiesis, PHZ-induced anemic frogs, which showed accelerated

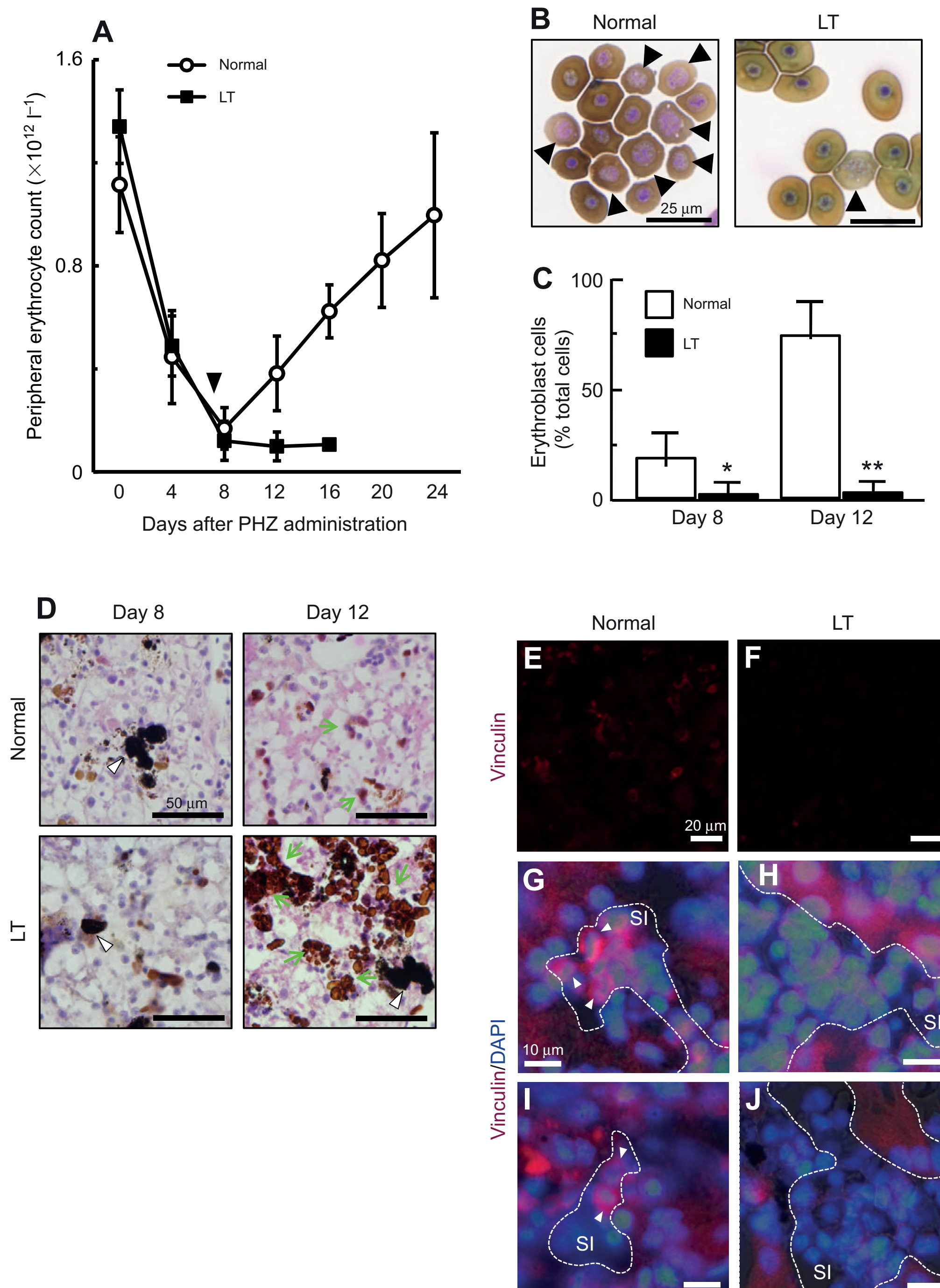

Fig. 4. The effect of low temperature on erythropoietic stress and distribution of newly produced erythrocytes. (A) Phenylhydrazine (PHZ; 25 mg kg^{-1} body mass) was used to induce anemia in frogs and peripheral erythrocytes were counted at 4 day intervals. Arrowhead indicates the start of low-temperature exposure (LT) on day 7 after PHZ administration. (B) Photomicrographs of blood smears from normal and low-temperature frogs on day 12 stained with *o*-dianisidine and Giemsa. Arrowheads indicate erythroblasts in which the nuclear:cytoplasmic ratio is larger than in mature erythrocytes. (C) The number of erythroblasts in total peripheral blood cells on days 8 and 12 (N=3; **P*<0.05, ***P*<0.01 compared with normal). (D) Paraffin-embedded section stained with *o*-dianisidine and Hematoxylin–Eosin. Hemoglobinized erythrocytes (brown/orange stained) appeared on day 12 (green arrows). Many more hemoglobinized erythrocytes were observed at low temperature than at normal temperature. Arrowheads indicate pigmented cells. (E–J) Cell adhesion between erythrocytes and sinusoid wall. Immunohistochemistry using anti-vinculin antibody in PHZ-induced anemic liver (day 12). Vinculin signals (red) were detected at normal temperature (E), but not at low temperature (F). (G,H) At normal temperature, vinculin-positive blood cells appeared on the surface of the sinusoid wall. (H,J) At low temperature, no blood cell interaction with the sinusoid wall was detected. Arrowheads indicate vinculin-positive blood cells. Broken lines indicate the surface of the sinusoid wall. SI, sinusoid area.

erythropoiesis, were exposed to low environmental temperature. At 22°C, peripheral erythrocyte count reached a nadir on day 8 after PHZ administration, and increased gradually thereafter. When frogs were exposed to low temperature from day 7 after PHZ administration, erythrocyte count remained suppressed from day 8 (Fig. 4A) and after day 12 five of the six frogs died. This was likely due to the induced anemia and low environmental temperature. We observed the presence of many erythroblasts in the peripheral blood on days 8 and 12 after PHZ administration. In the present study, few erythroblasts were observed at low temperature (Fig. 4B,C and Fig. 5D). By histological analysis using *o*-dianisidine, hemoglobin-positive erythrocytes were not found on day 8, but a few were observed on day 12 at 22°C; they were also absent at low temperature on day 8 but became abundant on day 12 (Fig. 4D). To examine adhesion of retained erythrocytes to the hepatic sinusoid wall, we stained tissues for the focal adhesion protein vinculin. Vinculin signals were detected at ambient temperature (Fig. 4E) and vinculin-positive blood cells were localized to the sinusoid wall (Fig. 4G,I); in contrast, no signals were found at low temperature (Fig. 4F,H,J). To examine the distribution of newly produced erythrocytes, we performed BrdU pulse labeling of frogs on day 7 after PHZ administration, and detection of BrdU-positive cells in liver and peripheral blood on day 12 by immunochemistry (Fig. 5A). BrdU-positive cells were as abundant in liver at 22°C as at low temperature (Fig. 5B,C). In peripheral blood, about half of all cells were BrdU positive at ambient temperature (Fig. 5D,E) while at low temperature

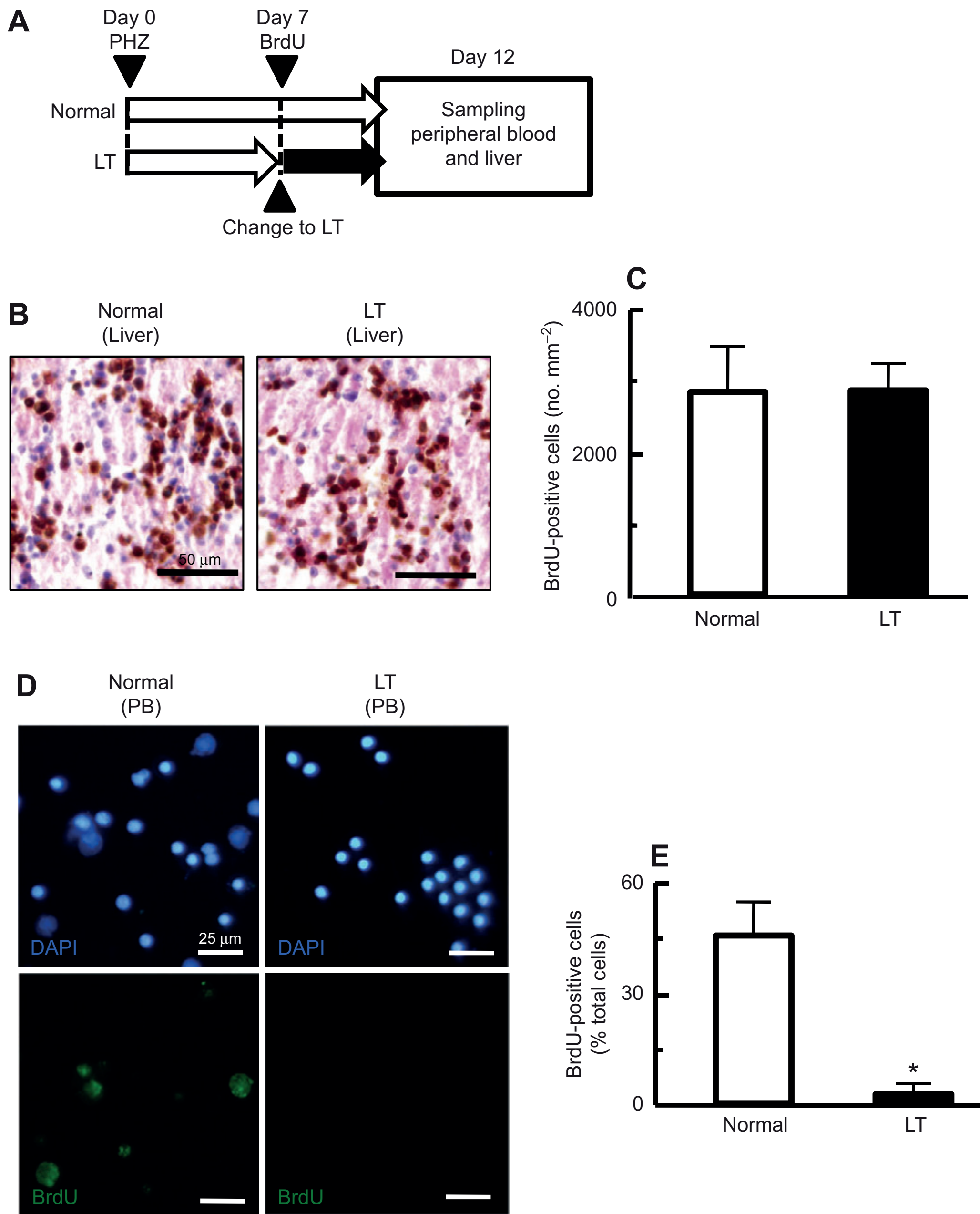

Fig. 5. *In vivo* erythrocyte proliferation assay using bromodeoxyuridine (BrdU). (A) BrdU was injected before exposure to low temperature on day 7 after PHZ administration. On day 12, samples of liver and peripheral blood were taken. (B) BrdU staining of liver sections. (C) Number of BrdU-positive cells (N=3 frogs). (D) BrdU staining of peripheral blood cells. Total cells detected by DAPI (upper panels) and BrdU-positive cells detected by Alexa Fluor 488 (bottom panels). (E) Percentage of BrdU-positive cells in peripheral blood cells (N=3; *P<0.01 compared with normal temperature).

the number was markedly decreased (2%). These results indicate that newly produced erythrocytes do not migrate from liver to peripheral blood at low temperature.

DISCUSSION

Half a century ago, Cline and Waldmann demonstrated that exposure to low temperature induced anemia and reduced erythropoiesis in leopard frogs (Cline and Waldmann, 1962). However, a relationship between the causes of anemia and erythropoiesis under hypothermic conditions has not been proposed before. In the current study, we induced anemia in *X. laevis* by low-temperature exposure and investigated the effects of hypothermic conditions on hepatic erythropoiesis. One day after exposure to 5°C, peripheral blood cell count decreased and then remained low for 5 consecutive days (Fig. 1D–F). Because the rate of decline of blood cell count was different among blood cell types (erythrocytes, leukocytes and thrombocytes), it was unlikely that the cause of cytopenia was an expansion of circulating plasma volume. Indeed, we could not determine significant change in circulating plasma volume between normal and low-temperature conditions by the Evans Blue dye dilution technique (data not shown).

In ectothermic animals, metabolic activity decreases at low temperature, and excessive erythrocytes and transfer of O_2 appear to cause hyperoxia and hypertension (González-Alonso et al., 2006). In the American toad (*Bufo americanus*), gas exchange rate decreases to approximately 5% of normal levels at 5°C (Hutchison et al., 1968). The fall in peripheral blood erythrocyte count observed in the present study may occur because a lower oxygen-carrying capacity is required by frogs under hypothermic compared with normal conditions, and is thus important in maintaining proper tissue oxygen tension during hypothermia. Hematocrit levels, one of the determining factors for blood viscosity, were significantly decreased in frogs under low-temperature exposure, compared with normal frogs (Table 2). Herman and Mata (Herman and Mata, 1985)

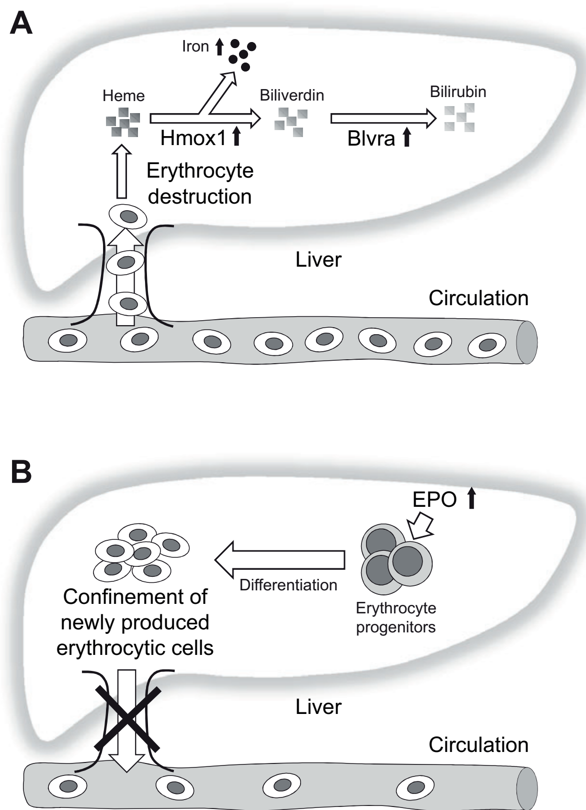

Fig. 6. Schematic models of *Xenopus laevis* hepatic erythropoiesis during low-temperature exposure. Low temperature induces peripheral erythrocyte degradation in the liver. Increased Hmox1 and Blvra expression leads to conversion of heme, originating from destroyed erythrocytes, to bilirubin, and then iron accumulation in liver (A). Subsequently, EPO produced in lung and liver stimulates proliferation and differentiation of erythrocyte progenitors in the liver. However, these newly produced erythrocytes are confined to the liver and do not migrate into the circulation. Hence, the frog remains anemic during low-temperature exposure (B).

reported that blood pressure decreases initially in the American bullfrog (*Rana catesbeiana*) during 5°C acclimation. Therefore, the risk of damaging circulating erythrocytes could be high. The fall in hematocrit observed may serve to decrease blood viscosity and the risk of damaging erythrocytes under hypothermic conditions. Although the cause of hypothermic leukocytopenia and thrombocytopenia could not be resolved in the current study, the rapid (1–2 days) response of blood cell count to a change in environmental temperature, from 22 to 5°C or the reverse, suggests that abnormality of hematopoietic stem cells and progenitor cells is not the direct cause of the pancytopenia. When blood platelets are refrigerated prior to transfusion, the clearance of platelets from the circulation is enhanced by the glycan–lectin mechanism (Hoffmeister, 2011). It is possible that such a lineage-specific reaction dependent on low temperature is one of the causes of hypothermia-induced thrombocytopenia and leukocytopenia in *X. laevis*. We are currently preparing methods for hematological analysis of *X. laevis* to help resolve these issues.

Our data indicated that hypothermic anemia was initiated by enhanced peripheral erythrocyte destruction. In adult *X. laevis*, histological observation suggested that liver and spleen are also the sites of phagocytosis and destruction of damaged erythrocytes (Chegini, et al., 1979). Several studies have reported instances of enhanced Hmox1 and Blvra expression. During erythrophagocytosis, *hmox1* mRNA levels in macrophages increased in an *in vitro* system (Knutson et al., 2003). Scavenger receptor class B, type I-deficient mice showed enhancement of *hmox1* and *blvra* mRNA levels in spleen and liver compared with wild-type mice due to the short lifespan of erythrocytes and enhanced erythrocyte destruction (Meurs et al., 2005). We have shown here that expression of *hmox1* and *blvra* in the liver was up-regulated 0.5 days (10-fold) and 1 day (38-fold) after exposure to low temperature (Fig. 2C). In the spleen, the expression of *hmox1* and *blvra* increased on day 1 after low-temperature exposure (Fig. 2D). Accumulation of iron, as a result of heme degradation, was detected in the liver but not in the spleen (Fig. 2E). The organ mass of the spleen is 1/47 that of the liver in adult *X. laevis* (0.35 mg g^{-1} body mass for spleen, and 16.4 mg g^{-1} body mass for liver). Collectively, our results indicate that low temperature induces erythrocyte destruction in the liver, and triggers enhancement of *hmox1* and *blvra* mRNA expression to catalyze heme degradation and reduce biliverdin to bilirubin. The contribution by the spleen to erythrocyte destruction is low compared with liver; hence, the spleen may play a subsidiary role in erythrocyte destruction induced by low-temperature exposure.

It has been reported that low environmental temperature down-regulates erythropoiesis in leopard frogs and zebrafish (Cline and Waldmann, 1962; Kulkeaw et al., 2010). However, in contrast, in our *Xenopus* model, we found that *epo* mRNA expression was elevated in lung and liver 1 day after low-temperature exposure and hepatic erythropoiesis was up-regulated (Fig. 3). Moreover, in another model of anemia, the PHZ-induced anemic model, new erythrocytes were also observed to be produced in the liver during low-temperature exposure (Figs 4, 5). Thus, in our hands at least, the cause of observed anemia in *X. laevis* under hypothermic conditions is not necessarily a down-regulation of erythropoiesis. However, despite up-regulation of erythropoiesis, newly produced erythrocytes are not released into the circulation but appear to remain in the hepatic sinusoid (Figs 4, 5), which would explain the prolonged anemia observed during hypothermia. Previously, the rate of erythropoiesis during hypothermia has been measured by peripheral erythrocyte incorporation of Fe^{59} (Cline and Waldmann, 1962). Although not examined by Cline and Waldmann, we found that newly formed erythrocytes incorporating Fe^{59} were distributed within hematopoietic tissue in leopard frogs during low-temperature exposure.

In mammalian bone marrow, sinusoidal endothelial cells have been reported to act on hematopoietic stem cells in a so-called progenitor-cell niche (Yin and Li, 2006), and in mouse fetal liver hematopoietic stem cells reside in the perisinusoid niche in a slowly cycling state (Iwasaki et al., 2010). We therefore initially hypothesized that erythrocytes adhered to the hepatic sinusoid and were confined there during low-temperature exposure. Vinculin staining indicated that erythrocyte interaction with the sinusoid wall was absent at low temperature (Fig. 4F,H,J). Therefore, confinement of new erythrocytes in the liver during low-temperature exposure may be independent of adhesion molecule interactions with the sinusoid. Other factors might be responsible for confining erythrocytes to the hepatic sinusoid. In 5°C-acclimated American bullfrogs, blood pressure and heart rate were reduced compared with normal frogs (Herman and Mata, 1985). Thus, it is conceivable that newly produced erythrocytes could not migrate from hepatic sinusoids to the circulation periphery due to the decrease in plasma blood flow. Hepatic vasoconstriction could also affect the release of newly produced erythrocytes from the production site during low-temperature exposure. It has been reported that the cardiovascular

effect of catecholamines is different between warm- and cold-acclimated bullfrogs (Herman and Mata, 1985). Endothelin-1 and nitric oxide regulate vascular diameter in *Xenopus* tadpoles (Schwerte et al., 2002). Thus, it is possible that hepatic vasoconstriction induced by vascular regulating factors acted locally to interfere with the release of newly produced erythrocytes from the liver to the circulation during low-temperature exposure.

Why was EPO elevated while newly produced erythrocytes were retained in the hepatic sinusoid during low-temperature exposure? One possibility is that excessive accumulation of iron in the liver induced erythropoiesis as a result of reduced hemosiderin and an imbalance in iron homeostasis. Patients with hereditary hemochromatosis have a systemic iron overload due to duodenal hyperabsorption that leads to an increase in hemoglobin, mean corpuscular erythrocyte volume and mean corpuscular hemoglobin content (Barton et al., 2000). Recovery of erythrocyte count 2 days after we returned the frogs to normal temperature (Fig. 1D) led us to hypothesize that erythrocytes are pooled for rapid release into the circulation once temperature normalizes. Proliferation and differentiation of erythrocytes are tightly regulated processes. In *X. laevis*, colony forming unit–erythroid (CFU-E) colonies arising from hepatic blood cells during 2 days of semisolid culture, differentiate a few days later into mature erythrocytes (Nogawa-Kosaka et al., 2010). So, hepatic pooling of erythrocytes may be required for adaptation to a wide range of temperatures in *X. laevis*.

In conclusion, we have shown that anemia can be induced in *X. laevis* by exposure to an environmental temperature of 5°C. Acute exposure to low temperature leads to the destruction of erythrocytes in the liver (Fig. 6A). Despite evidence of erythropoiesis in response to anemia, newly produced erythrocytes were confined to the liver, and erythrocyte count remained low throughout the experimental time period (Fig. 6B). This experimental model offers a new approach to investigating the mechanisms of anemia response under hypothermic conditions in vertebrates. Moreover, the hematopoietic response to environmental temperature change described here may lead to new insights into vertebrate hematology.

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