Review Article

Diverse of Erythropoiesis Responding to Hypoxia and Low Environmental Temperature in Vertebrates

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Received 28 April 2015; Accepted 4 September 2015

Academic Editor: Hasan Mahmud

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Erythrocytes are responsible for transporting oxygen to tissue and are essential for the survival of almost all vertebrate animals. All vertebrates adapt to various environments. Therefore, investigations of erythropoiesis in vertebrates are considered vital to address the various erythropoietic responses to environmental stress. The vertebrates that lack hemoglobin and erythrocytes are the larvae of eels and a few Antarctic fish of the family Channichthyidae [1]. Moreover, only mammalian species in vertebrates have anucleate erythrocytes, while erythrocytes of nonmammalian species have nuclei and a shape of an ellipse. The advantage of anucleate erythrocyte is explained by flexibility in developed capillary and incensement of surface area for oxygen binding. Moreover, it has been demonstrated that the erythropoietic systems are diverse among vertebrates (Table 1). Although the science behind them is less than clear-cut, such a wide-ranging difference should bring diversity to the hematopoietic systems for responding to environmental stress. To address the description of erythropoietic functions, this paper introduces the present understanding of erythropoiesis in vertebrates, focusing on erythropoietic responses to environmental stress, hypoxia, and lowered temperature.

1. Introduction

Erythrocytes are responsible for transporting oxygen to tissue and are essential for the survival of almost all vertebrate animals. All vertebrates adapt to various environments. Therefore, investigations of erythropoiesis in vertebrates are considered vital to address the various erythropoietic responses to environmental stress. The vertebrates that lack hemoglobin and erythrocytes are the larvae of eels and a few Antarctic fish of the family Channichthyidae [1]. Moreover, only mammalian species in vertebrates have anucleate erythrocytes, while erythrocytes of nonmammalian species have nuclei and a shape of an ellipse. The advantage of anucleate erythrocyte is explained by flexibility in developed capillary and incensement of surface area for oxygen binding. Moreover, it has been demonstrated that the erythropoietic systems are diverse among vertebrates (Table 1). Although the science behind them is less than clear-cut, such a wide-ranging difference should bring diversity to the hematopoietic systems for responding to environmental stress. To address the description of erythropoietic functions, this paper introduces
| Species                          | EPO Producing organ | Erythropoietic organ Site                  | Cell identification                  | Days | Cell labeling                       |
|---------------------------------|---------------------|-------------------------------------------|--------------------------------------|------|--------------------------------------|
| **Fish**                        |                     |                                           |                                      |      |                                      |
| Pufferfish (Takifugu rubripes)  | Heart [8]           |                                           |                                      |      |                                      |
| Zebrafish (Danio rerio)         | Heart [9, 10]       | Kidney                                    | gata1 reporter transgenesis [26, 27], progenitor assay [29] |      |                                      |
| Brown trout (Salmo trutta)      | Spleen, kidney      |                                           | Cell morphology [19]                 |      |                                      |
| European perch (Perca fluviatilis)| Spleen             |                                           | Cell morphology [19]                 |      |                                      |
| Common Roach (Rutilus rutilus)  | Kidney              |                                           | Cell morphology [19]                 |      |                                      |
| Common carp (Cyprinus carpio L) | Kidney              |                                           | Cell morphology [25]                 |      |                                      |
| Ginbuna crucian carp (Carassius auratus langsdorffii)| Kidney (stem cell) | Kidney marrow cell transplantation [81] | 270 | PKH 26-GL [40]                      |
| **Amphibian**                   |                     |                                           |                                      |      |                                      |
| African clawed frog (Xenopus laevis) | Lung, liver [12]  | Liver                                     | Progenitor assay [30], immunohistochemistry [14] | 220  | Biotin in vivo [39]                 |
| Bullfrog (Lithobates catesbeianus) | Kidney, bone marrow |                                           | Immunohistochemistry [31]            |      |                                      |
| Leopard frog (Lithobates pipiens) |                   |                                           |                                      | 200  | DFP32 [82]                         |
| Great crested newt (Triturus cristatus) | Spleen, heart      |                                           | Cell morphology [20], PHZ-induced anemia [23] |      |                                      |
| Ezo salamander (Hynobius retardatus) | Spleen             |                                           | In situ hybridization [83]           |      |                                      |
| **Reptiles**                    |                     |                                           |                                      |      |                                      |
| Painted turtle (Chrysemys picta) | Bone marrow, kidney, spleen |                           | Fe^{59} incorporation [32]             |      |                                      |
| European pond turtle (Emys orbicularis) | Bone marrow |                                           | Cell morphology [21]                 |      |                                      |
| Spanish lizard (Lacerta hispanica) | Bone marrow         |                                           | Cell morphology [22]                 |      |                                      |
| Boa constrictor (Boa constrictor) | Bone marrow         |                                           | Cell morphology [24]                 |      |                                      |
| Corn snake (Elaphe guttata)     | Bone marrow         |                                           | Cell morphology [24]                 |      |                                      |
| Brown house snake (Lamprophis fuliginosus) | Bone marrow |                                           | Cell morphology [24]                 |      |                                      |
| Bothrops jararaca (Bothrops jararaca) | Bone marrow, spleen |                                           | Cell morphology [24]                 |      |                                      |
| **Avian**                       |                     |                                           |                                      |      |                                      |
| Chicken (Gallus gallus)         | Bone marrow         |                                           | Progenitor assay [33]                | 35   | Na$_2$Cr$_{51}$O$_4$ [38]            |
| White Carneaux pigeons (Columba livia) |                  |                                           |                                      | 35–45| Na$_2$Cr$_{51}$O$_4$ [38]            |
| White Pekin duck (Anas platyrhynchos) |                  |                                           |                                      | 42   | Na$_2$Cr$_{51}$O$_4$ [38]            |
the heart [8]; however, the biological function of the heart
EPO has not been directly elucidated. We have reported the
identification and biological properties of EPO [11, 12] and
EPO receptors (EPOR) [13, 14] in the African clawed frogs,
Xenopus laevis. The highest expression of EPO in Xenopus
laevis was found in the lung and lesser in the liver [12]. It
was our surprise that Xenopus EPO, unlike in human and
murine EPO, lacks N-glycosylation that is essential for in vivo
stability and biological activity in the circulation. When N-
linked carbohydrates were artificially introduced to Xenopus
EPO, however, in vitro activity was not interfered [15]. We
also demonstrated that recombinant Xenopus EPO induces
proliferation of human cell lines expressing Xenopus EPOR
as well as human EPOR, despite the 38% amino acid identity
shared between Xenopus EPO and human EPO [12]. In
addition, an essential tertiary structure of the ligand-receptor
(EIFP-EPOR) is reserved and shared among those species [11].
Because of these findings, the ligand specificity should not be
determined solely by primary amino acid sequences.

2.2. Erythropoietic Organ. Erythropoietic organs in verte-
brates are various. In mammals, erythropoiesis mainly occurs
in bone marrow at their adult stage. Additionally, the spleen
also causes erythropoiesis in some rodents [16–18]. The stud-
ies of searching hematopoietic organs in nonmammals have
been performed through the ages [19–25]. Although most
investigations are approached though cellular morphological
observation, there are reports using molecular based technics
during recent years. In teleost fish, hematopoiesis occurs in the
kidney. A study of brown trout has indicated that the spleen is also
an erythropoietic organ [19]. In zebrafish study, reporter
transgenesis takes the advantage to isolate and characterize
the hematopoietic progenitor cells. An approach using gata1
reporter transgene and flow cytometry to separate the ery-
throblastic cells from the kidney has been developed in the
gata1 reporter transgenic models [26–28]. Kidney of zebrafish
comprehends the erythroid progenitors that induced proliferation and differentiation by recombinant EPO in vitro
[29].

In aquatic amphibian, Xenopus laevis, we recently demon-
strated that erythropoiesis mainly occurs in the adult liver
to reveal the presence of CFU-E [14, 30]. The EPOR positive
cells are found predominantly on the inner wall of hepatic
sinusoids and increased during erythropoietic stress [14]. In
northern crested newt, erythroblastic cells are observed in the
heart aside from the spleen after phenylhydrazine- (PHZ-)
induced anemia. Although we also observed erythroblasts in
periphery during erythropoietic stress induced by PHZ, ery-
throblasts could not be observed in periphery in normal state
[13]. Therefore, it is suggested that these erythroblast cells that
are observed in periphery are released from erythropoietic
organ responding to erythropoietic stress.

While, in bullfrog (Lithobates catesbeianus), terrestrial
amphibian, de Abreu Manso and his colleagues reveal that
hematopoietic cells expressing CD34, CD117, and EPOR exist
in the adult bone marrow of vertebrate, femur, and fingers
and in the kidney detected by immunohistochemistry [31],
in reptiles and avian, bone marrow is the primary erythropoi-
etic site [21, 22, 24, 32, 33]. In chicken, erythroid progenitor
assay is developed well using bone marrow cells [33]. Taken
together, in aquatic amphibian, erythropoiesis mainly occurs
in kidney, spleen and/or liver. From terrestrial amphibian,
erythropoiesis is found in bone marrow, while, in aquatic
mammalian bottlenose dolphins (Tursiops truncatus), bone
marrow mononuclear cells contain hematopoietic progenitor
cells [34].

2.3. Fate of Circulating Erythrocytes. Erythrocyte is damaged
little by little during circulation and destructed eventually.
In adult human, the life span of erythrocyte is about 120
days [35]. The life span is not correlating with the direction of
evolution. The erythrocytes lifespans of mice [36], rabbit
[37], and chickens [38] are about 40, 55, and 35 days,
respectively, which are shorter than human. In amphibian
and fish, the life span tends to be longer compared with
mammalian species. The life span of erythrocytes in Xeno-
pus laevis is about 220 days [39], longer than mouse and
human. In ginpuna crucian carp, the erythrocyte lifespans is
about 50 days, and labeling erythrocytes are detected in
circulation up to 270 days [40]. Biological implication of
difference in the erythrocyte life span is not well
understood.

In mammals, damaged or senescent erythrocytes are
degraded by phagocytes in the liver and spleen [41]. Investi-
gations of erythrocyte destruction sites in nonmammalian
species are limited. Our recent work with Xenopus laevis
showed that erythrocyte destruction mainly occurred in the
liver based on the expression of heme degrading enzymes,
hemeoxygenase-1, and biliverdin reductase A expression and
the accumulation of iron [39].

3. Erythropoiesis Response to
Environmental Stress

3.1. Hypoxia. The primary EPO producing organ is the
kidney in adult mammal, while most of the knowledge of
hypoxic EPO expression has been based on human hepatoma
cell lines. The molecular mechanisms of hypoxia inducible
EPO gene expression involve hypoxia inducible transcription
factors (HIFs), which are primary transcriptional factors of
the hypoxic response [7, 42] and are negatively regulated by
the von Hippel-Lindau (VHL) factor [43, 44]. The VHL
acts to ubiquitylate the catalytic α subunit of HIF and
induce their turnover in normoxia due to recognition of
a prolyl hydroxylation motif, which is oxygen dependent
hydroxylases to modify α subunit of HIF. During hypoxic
condition, the loss of hydroxylase activity causes the accumu-
lation and transactivation function of HIF-α subunit
[45]. The most well characterized HIF regulatory region is a
liver-specific hypoxia response element (HRE) that is
the binding site of HIF-1, which is located within 0.7 kb 3’
of the polyadenylation signal [45]. Recently the analysis of
renal EPO expression has been performed and increasingly
obvious [46–48]. There are also reports that EPO is detected
in various tissues (brain, heart, lung, and testis) and exhibited
nonerythropoietic activity [49–51]. Taken together, we have a question if each EPO producing organ could response to hypoxia and promote erythropoiesis or not. To resolve this issue, conditional knockout experiments in which a target gene can be specifically inactivated in specific tissue(s) were performed. The deletion of VHL gene prevents the ubiquitination of hydroxylated HIF-α proteins and causes a hypoxic phenotype due to accumulation of HIF-α proteins. To demonstrate the contribution of each EPO producing organ, various mice in which VHL can be inactivated tissue specifically were generated using conditional gene targeting technology based on Cre-loxP mediated recombination [52]. Hepatocytes specific deletion of VHL using albumin promoter enhanced hepatic EPO expression and induced polycythemia [53]. Mice with osteoblasts specific knockout of VHL showed the enhancement of EPO expression in bone marrow and erythropoiesis [54]. The expression level of renal EPO was decreased in the mice. Interestingly, it has been reported that renal EPO expression is induced by the variation of oxygen partial pressure in other tissues. Rats with high cerebral pressure exhibit a significant increase in plasma EPO levels [55]. This report suggested that EPO expression in the kidney is induced by a brain stem-derived humoral factor at high intracranial pressure. Boutin et al. showed that HIF-1α gene deletion in mice epidermis inhibits renal EPO synthesis in response to hypoxia and epidermal sensing of oxygen is important for the renal EPO synthesis [56]. Based on these observations, it is suggested that each organ have an oxygen sensor independently in mammalian species.

In nonmammalian species, the knowledge of EPO gene expressions responding to hypoxia is limited. In zebrafish, cardiac EPO mRNA expression is moderately upregulated 6.5 h after being exposed at hypoxic environment [9]. HRE in teleost EPO gene locus of fugu has been reported [57]. The fugu HRE is located in the 5' flanking region of EPO gene locus. However, the HRE is on the opposite strand of DNA, unlike the HRE of EPO gene locus in the human, and it is unclear whether the HRE contributes to response to hypoxia and induces the EPO gene expression or not. We recently have showed that the expression of EPO gene in Xenopus laevis is not induced by anemia investigated [12]. The hypoxic response and diversity organs of EPO gene expression in nonmammalian species are subject of future investigation.

Before indicating the existence of EPO, Misher (1893) suggested that hypoxic stress directly stimulates bone marrow and promotes erythropoiesis. Although this hypothesis was rejected by the identification of EPO once, the response of erythroid cells to hypoxia has been recently investigated. Ex vivo erythrocytes production from peripheral and cord blood CD34+ cells was enhanced by low oxygen concentration (1.5–5%) exposure [58]. Under hypoxic conditions, the mRNA and protein amounts of delta-aminolevulinate synthase 2 (ALAS2) and GATA-1 (erythroid specific transcriptional factor) were elevated in human erythroleukemic cell line, K562 cells, and erythroid induction cultures of CD34+ hematopoietic stem/progenitor cells [59, 60].

It has been reported that the type of hemoglobin is changed by hypoxia stimuli. In human, embryonic, fetal, and adult hemoglobin is sequentially expressed in erythroblasts during developing developments. The binding affinity of hemoglobin is different among the type of hemoglobin. Increasing the oxygen-binding affinity of fetal hemoglobin is relative to that of adult. In erythroid differentiated culture from human hematopoietic progenitor cells, the number of cells with fetal hemoglobin increased, exposed to low O2 [61]. A moderate increase of circulating erythrocytes with fetal hemoglobin was observed in human during and after the hypoxia exposure [62]. Erythrocytes with fetal hemoglobin have an advantage to bind more oxygen under hypoxic condition. In amphibian species, hemoglobin switch from the larval to the adult type of hemoglobin has been showed during metamorphosis (from aquatic to terrestrial life) [63, 64]. Because the oxygen dissociation curve in tadpole shifts to left compared with that in adult [65], the tadpole erythrocyte more readily takes up oxygen, so that it is an advantage for tadpoles to live in water with lower oxygen. These reports indicated that hemoglobin switching is one of the important responses to adapt hypoxic environment.

Recently, a type of noncoding RNA, micro-RNA (miRNA), has attracted attention for the factor responding to environmental stress. miRNAs are single-stranded RNA molecules approximately 22 nucleotides in length. Each miRNA recognizes target mRNAs via base-pairing interactions and induces the gene silencing. We compared the miRNA expression patterns in UT-7/EPO, UT-7/GM, and UT-7/TPO cell lines which are all differentiated from UT-7 cells by cytokine stimulation and exhibit the same genotype with different phenotypes, and demonstrated some specific miRNAs expressed in erythroid cells [66]. One of them, miR-210, is expressed in the late stage erythroid cells in mouse [66]. miR-210 is highly expressed by hypoxic condition and regulates the iron homeostasis via targeting the iron-sulfur cluster scaffold protein and transferrin receptor [67]. Recently, Sarakul et al. demonstrated that miR-210 induced erythroid differentiation in K562 cells and CD34+ hematopoietic stem/progenitor cells [68]. Although seen mostly in vitro, these results suggested that erythroid cells could be regulated by low O2 condition beyond the EPO response.

3.2. Low Temperature. In birds and mammals, endothermic animals process the mechanisms to maintain the body temperature, independent of the environmental temperature. Upon the initial exposure to low temperature, endothermic animals exhibit peripheral vasoconstriction to reduce heat loss from body. Active thermogenesis occurs by means of periodic shivering if heat dissipation exceeds metabolic heat generation [69]. During prolonged exposure to low temperature, nonshivering thermogenesis is enhanced by synthesis and catalysis of ATP in the brown adipose tissue and muscle. Additional metabolic heat generation is accompanied by increased oxygen consumption [69]. Therefore, it is readily hypothesized that erythropoiesis would
respond and contribute to adaptation to low environmental temperature.

There are many reports that showed the response of hematopoiesis to environmental temperature. Nine-banded armadillo (Dasypus novemcinctus) possesses the active hematopoiesis in the dermal bone marrow of the armor. In winter season, the dermal bone marrow became fatty and showed less hematopoiesis, compared with summer season [70]. The marrow of tailbone in new born rats has active hematopoiesis and replaced the adipose with maturity. Tavassoli et al. showed that the capability of hematopoiesis was maintained in the tail vertebrae of newborn rats by transposing the tail into the warmer environment of the abdomen [71]. It has been demonstrated that the variation of blood cell counts is induced by low environmental temperature and season change in various vertebrates (Table 2). In rats and chickens acclimated to low temperature, an increase in the number erythrocytes was observed [72, 73]. We have investigated whether increase in circulating erythrocytes induced by low temperature exposure is caused by enhanced erythropoiesis in mice [74]. Mice were exposed to a 5°C environment for 56 days. The blood hematocrit levels (HCT) gradually increased by day 14 and remained high at day 56. The proportion of proerythroblasts in the spleen and bone marrow increased greatly by day 5 of exposure to low temperature. EPO mRNA levels increased in the kidneys, and hypoxia inducible genes were enhanced in the kidney after being exposed to low-temperature environment. These results indicated that erythropoiesis was enhanced, explaining the high level of EPO mRNA expression in the kidney. In addition, the level of oxygen tension in the kidney was decreased after low-temperature exposure. Elevated erythrocyte counts would increase oxygen supply to peripheral tissues for heat production after low-temperature exposure.

In the studies of nine-banded armadillo and newborn rats as described previously, it is considered that hematopoietic effects are caused by hematopoietic organ itself exposed to low temperature. Moreover, cases of anemia or pancytopenia in hypothermic patients have been reported [75–79]. Establishing the hypothermic model in small mammals has been hampered by practical difficulties. We used the African clawed frog, Xenopus laevis was used to investigate the cause of hypothermia-induced anemia [39]. Frogs were exposed to low temperature (5°C) for five days and then were put back to 22°C immediately afterwards. One day after exposure to 5°C, erythrocyte count decreased by approximately 30% and then remained at this level for 5 days. Two days after the return to 22°C, erythrocyte count had recovered to initial levels. The rate of destruction of erythrocytes in adult Xenopus laevis was estimated to be about 0.45% per day under normal conditions from 220-day erythrocyte lifespan. It was hypothesized that enhancement of erythrocyte degradation caused erythrocytopenia after low-temperature exposure. Primary organ of erythrocyte degradation is the liver in adult Xenopus laevis. In the liver, heme oxygenase (HOMX1) and biliverdin reductase (BLVRA) mRNA increased after exposure to low temperature, and then accumulation of iron as a result of heme degradation was observed in the liver. These results indicated that hypothermic anemia was initiated by enhanced peripheral erythrocyte destruction. It is hypothesized that the cause of anemia under hypothermic conditions is a downregulation of erythropoiesis. However, in contrast, we found that EPO mRNA expression was elevated in lung and liver after low-temperature exposure and hepatic erythropoiesis was upregulated. Despite upregulation of erythropoiesis, newly produced erythrocytes are not released to the circulation but appear to remain in the hepatic sinusoid, which could explain the prolonged anemia observed during low-temperature exposure. To investigate the modulated molecules responding to low temperature, moreover, we attempted the proteomics to profile the hepatic proteome in Xenopus laevis after exposure to low temperature [80]. Our proteome data suggested that glycolytic and antioxalate systems acted after the accumulation of hepatic iron caused by low-temperature exposure.

The signal passway from sensitive environmental low temperature to erythropoietic change is unclear. However, we demonstrated that the rule of erythropoiesis responding to low environmental temperature is different between endothermic and ectothermic animals through our comparative study.

4. Conclusion and Perspective

Since the discovery of EPO, it has been investigated that the expression and secretion of renal EPO which respond to environment and tissue oxygen tension could regulate the circulating erythrocyte counts. Through the study of various vertebrates, it has become increasingly clear that vertebrates possess the unique erythropoietic responses to habitat and environmental stress. Recently, the many experimental technologies, such as gene editing and omics, have been developed and are available to not only laboratory animals but also nonlaboratory animals. Therefore, comparative study holds the more potential for enhancing our knowledge of erythropoietic systems. Furthermore, to understand the physiology of the whole organism and/or cell, analyzing cyclopedic modulating molecules induced by environmental stress and understanding the network relationship among these molecules were attempted. Through this analysis, the various systems to link to erythropoiesis will become known.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper. No financial interests/relationships with financial interest relating to the topic of this paper has been declared.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science; by Strategic Foundation Grant-Aided Project for
Table 2: Hematopoietic responses to low environmental temperature and seasons in vertebrates.

| Stress          | Species                                | Experimental condition | Peripheral blood cell counts | Hematopoietic observation                                                                 | Reference |
|-----------------|----------------------------------------|------------------------|------------------------------|-------------------------------------------------------------------------------------------|-----------|
| Seasonal change | Nine-banded armadillo                  | Winter                 | Increase, Decrease           | Decrease hematopoietic cells in armor dermal bone marrow                                  | [70]      |
|                 | Edible frog (Rana esculenta)           | Winter                 | RBC, HGB                     |                                             | [84]      |
|                 | Wisent European bison (Bison bonasus)  | Summer                 | RBC size                     |                                             | [85]      |
|                 | Grass snake (Natrix natrix)            | Winter                 | RBC, HGB, HCT                |                                             | [86]      |
|                 | Bottlenose dolphin (Tursiops truncatus)| Winter                 | RBC, HGB, HCT                |                                             | [87]      |
| Low temperature | Leopard frog (Rana pipiens)            | 5°C                    | HCT                          | RBC lifespan extends                       | [88]      |
|                 | Painted turtle (Chrysemys picta)       | 2°C                    | HCT                          |                                             | [89]      |
|                 | Cunningham's skink (Egernia cunninghami)| 8°C                    | HCT, HGB                     |                                             | [90]      |
|                 | Sidewinder (Crotalus cerastes)         | 20°C                   | RBC, HGB, HCT                |                                             | [91]      |
|                 | Fossil catfish (Heteropneustes fossilis)| 18°C                   | RBC, HGB, HCT                |                                             | [92]      |
|                 | American bullfrog (Rana catesbeiana)   | 5°C                    | RBC, WBC, TBC                |                                             | [93]      |
|                 | Rat (Rattus norvegicus)                | 5°C                    | RBC, HGB, HCT                |                                             | [72]      |
|                 | Chicken (Gallus domesticus)            | 10°C                   | HGB, HCT                     | Downregulate erythropoietic genes expression level                                          | [73]      |
|                 | Zebrafish (Danio rerio)                | 17°C                   | HGB, HCT                     | Enhanced hepatic erythrocyte destruction, Newly produced erythrocytes are confined to the liver | [94]      |
|                 | African clawed frog (Xenopus laevis)   | 5°C                    | RBC, WBC, TBC                |                                             | [39]      |
|                 | Mouse (Mus musculus)                   | 5°C                    | HCT, HGB                     | Upregulate erythropoiesis                    | [74]      |
| Other           | European hamster (Cricetus cricetus)    | Hibernation            | WBC, TBC                     |                                             | [95]      |
|                 | Syrian hamster (Mesocricetus auratus)  | Hibernation            | TBC                          |                                             | [72]      |
|                 | Rat (Rattus norvegicus)                | Transposing the tail into the abdomen | Maintain the hematopoiesis in bone marrow of tail vein |                                             | [71]      |

RBC, erythrocyte; HGB, hemoglobin; HCT, hematocrit; WBC, leukocyte; TBC, thrombocyte.

Private Universities from The Japanese Ministry of Education, Culture, Sports, Science, and Technology (MEXT) (2012–2017); and by Waseda University grants for special research projects. Part of this study was performed as a component of a Private University “High-Tech Research Center” project supported by MEXT (2007–2011).

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