Characteristic Distribution of Hematopoietic Cells in Bone Marrow of *Xenopus Laevis*

Sumiharu Morita¹, Takeshi Moriishi², Satoru Matsunaga¹,³, Kei Kitamura³,⁴, Shin-ichi Abe¹,³ and Akira Yamaguchi⁵

¹ Department of Anatomy, Tokyo Dental College, 2-9-18 Kanda-Misakicho, Chiyoda-ku, Tokyo 101-0061, Japan
² Department of Cell Biology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan
³ Tokyo Dental College Research Branding Project, Tokyo Dental College, 2-9-18 Kanda-Misakicho, Chiyoda-ku, Tokyo 101-0061, Japan
⁴ Department of Histology and Developmental Biology, Tokyo Dental College, 2-9-18 Kanda-Misakicho, Chiyoda-ku, Tokyo 101-0061, Japan
⁵ Oral Health Science Center, Tokyo Dental College, 2-9-18 Kanda-Misakicho, Chiyoda-ku, Tokyo 101-0061, Japan

Received 12 November, 2020/Accepted for publication 4 March, 2021
Published Online in J-STAGE 15 August, 2021

Abstract

Bone marrow is the principal site of hematopoiesis in mammals. Amphibians were the first phylogenetic group in vertebrates to acquire bone marrow, but the distribution of hematopoietic cells in the bone marrow of the primitive frog, *Xenopus laevis* (*X. laevis*) has not been well documented. The purpose of this study was to perform a histological investigation of the distribution of hematopoietic cells in femoral bone marrow at various stages of development in *X. laevis*. Hematopoietic cells showed preferential distribution on the endosteal surface of cortical bone throughout all stages of development, from tadpole to aged frog. In mature frogs, hematopoietic cells appeared at the boundary between the epiphysis and the bone marrow. The distribution of hematopoietic cells around the blood vessels was limited to a small number of vessels in the bone marrow. Abundant adipose tissue was observed in the bone marrow cavity from the tadpole stage to the mature frog stage. Hematopoietic cells showed preferential distribution in a belt-like fashion on the surface of newly-formed bones in a bone regeneration model in the diaphysis of *X. laevis*. These results indicate that the distribution of hematopoietic cells in bone marrow in *X. laevis* differs from that in mammals, and that the bone marrow of *X. laevis* constitutes a useful model for exploring the mechanism underlying the phylogenetic differentiation of bone marrow hematopoiesis.

Key words: Hematopoiesis — Bone marrow — Blood vessel — Adipocyte — *Xenopus laevis*
**Introduction**

Bone marrow plays a major role in hematopoiesis in mammals. In humans, hematopoiesis occurs exclusively in the bone marrow. However, extramedullary hematopoiesis, which is hematopoiesis outside of the bone marrow, is also known to occur infrequently at other locations, including the spleen and liver, under pathological conditions such as myeloproliferative syndrome, chronic myelogenous leukemia, and aplastic anemia. Although bone marrow is the key hematopoietic organ in rodents, extramedullary hematopoiesis also occurs in the yolk sac, liver, and spleen at the fetal developmental stages, and continues to occur under physiological conditions in the spleen. In birds, hematopoiesis occurs mainly in the bone marrow and bursa of Fabricius, which is an organ specific to birds. In reptiles, it occurs in the bone marrow and spleen after birth. In amphibians, hematopoiesis occurs in the kidney, liver, spleen, and bone marrow, but the hematopoietic cells in each organ vary depending on the species. In fishes, hematopoiesis occurs in the kidney and spleen, as fishes lack a bone marrow cavity. Thus, depending on the phylogenetic group, hematopoiesis also occurs in various tissues other than bone marrow. Amphibians were the first phylogenetic group among vertebrates to acquire bone marrow. How the bone marrow gained active hematopoietic function in this phylogeny, however, remains to be fully determined.

Recent findings indicate that the bone marrow microenvironment containing the hematopoietic stem cell niche plays a crucial role in hematopoiesis. Two types of niche, the osteoblastic and the vascular, were reported to maintain and regulate hematopoietic stem cells in bone marrow. The osteoblastic niche mainly comprises osteoblastic cells, but some recent studies have reported a lack of concrete evidence linking them to the hematopoietic stem cell niche. The vascular niche consists of heterogeneous cell types in nature and origin such as sinusoidal endothelial cells, perivascular cells, and mesenchymal stromal cells, including reticular cells and adipocytes. Since blood vessels are essential for the vascular niche, an abundant supply of blood vessels in the bone marrow is indispensable to maintenance of its function. Thus, hematopoietic stem cell niches have been well studied in mammals, especially in mice and humans, but remain to be well documented during vertebrate phylogeny, including in *Xenopus laevis* (*X. laevis*).

There have been many reports concerning hematopoiesis in *X. laevis*, but they have predominantly focused on hematopoiesis during the embryonic stages and have not attempted to describe hematopoiesis in bone marrow in detail. This incomplete understanding of the process prompted us to explore and characterize the details of femoral hematopoiesis at various stages of development in *X. laevis*. This study may provide valuable information toward a more comprehensive understanding of the characteristics of hematopoiesis in bone marrow during vertebrate phylogeny.

The purpose of this study was to investigate the distribution of femoral hematopoietic cells at various developmental stages in *X. laevis*, from tadpole to aged frog. The results confirmed that preferential distribution of hematopoietic cells on the bone surface is a distinctive feature of the femur in *X. laevis*.

**Materials and Methods**

1. **Experimental animals**

Tadpole-stage, metamorphic, growing, and mature African clawed frogs (*X. laevis*) were purchased from Hamamatsu Seibutsu Kyouzai (Shizuoka, Japan) and were kept in aquaria at room temperature. The developmental stages of the tadpoles and frogs during the metamorphic and adult stages were determined using the stage classification (NF stage) proposed by Nieuwkoop and Faber (Fig. 1). The size of the growing and mature frogs was determined by measuring body length. At least 5 tadpoles or frogs were used.
to measure size and conduct histological examinations at each developmental stage. Femurs isolated from tadpoles and frogs were used for morphological observation. The representative histology at each developmental stage is described in the corresponding Figure below. All experimental procedures and protocols in the present study were approved by the Animal Research Ethics Committee of Tokyo Dental College (approval number: 285101).

2. Preparation of paraffin-embedded sections

The hindlimbs of X. laevis tadpoles and femurs of growing frogs were dissected under anesthesia with 0.1% ethyl-o-aminobenzoate (Wako, Osaka, Japan). Specimens were fixed in 4% paraformaldehyde (Wako) in phosphate buffered saline (PBS, pH 7.4) and used for histological examination.

The fixed samples obtained at various developmental stages were decalcified in 10% ethylenediaminetetraacetic acid (EDTA, Wako) at room temperature for 10 days. After being washed in PBS and dehydration in an ascending alcohol series, the samples were embedded in paraffin. Decalcified, 4-μm thick sections were prepared and stained with hematoxylin-eosin (Wako) and Giemsa for microscopic observation.

3. Bone regeneration model

Mature frogs (4 cm in size) were used for observation of hematopoiesis during bone regeneration. Surgery was performed under anesthesia with 0.1% ethyl-o-aminobenzoate (Wako). A 5-mm incision was made in the front skin of the mid-femur. After splitting the muscle, the periosteum was separated to expose the femoral surface. A drill-hole injury was created in the anterior of the diaphysis of the femur using a drill bit with a diameter of 0.5 mm. Two frogs were sacrificed at week 4 and 2 at week 6 after injury and the femurs (including the injury sites) dissected, fixed in 4% paraformaldehyde solution in PBS, and decalcified in 10% EDTA after washing with PBS. The samples were embedded in paraffin after dehydration in an ascending alcohol series. Paraffin sections (4-μm thick) were prepared and stained with hematoxylin and eosin for microscopic observation.

Results

1. Distribution of hematopoietic cells in femurs of tadpole and metamorphic X. laevis

At stage NF58, a late stage of development in X. laevis tadpole, mesenchymal cells resembling bone marrow mesenchymal cells and blood vessels appeared beneath the bone collar, but no hematopoietic cells were observed in the femur (Fig. 2A, B). Hematopoietic cells first appeared in the eroded cartilage beneath the bone collar at stage NF61 (Fig. 2C). At stage NF62, a middle stage of metamorphosis, hematopoietic cells were observed scattered
throughout the bone marrow at the mid-diaphysis (Fig. 2D, E). Hematopoietic cells appeared in clusters among bone marrow mesenchymal cells, including adipocytes (Fig. 2F). Some clusters of hematopoietic cells were observed adjacent to the bone surface (Fig. 2F).

2. Distribution of hematopoietic cells in growing and mature *X. laevis*

In the femurs of growing frogs (1 cm in size), the bone marrow had expanded in the
mesiodistal direction (Fig. 3A). Hematopoietic cells, which were observed scattered sparsely in the bone marrow, tended to be arranged close to the cortical bone (Fig. 3A–C). Blood vessels were observed scattered in the bone marrow, with some containing red blood cells. Hematopoietic cells were distributed more densely adjacent to the bone surface than around the blood vessels (Fig. 3B). Numerous adipocytes were evident in
In the femurs of growing frogs (2 cm in size), numerous hematopoietic cells located adjacent to the endosteal surface of cortical bone were arranged in a belt-like fashion (Fig. 3D–F). These cells were mainly composed of mononuclear cells with round-shaped nuclei (Fig. 3F). Mature adipocytes occupied most of the bone marrow space (Fig. 3E, F). Blood vessels were observed scattered throughout the bone marrow, but a few hematopoietic cells were visible around the vessels (Fig. 3E). A few hematopoietic cells were also noted at the boundary between the uncalcified cartilage and bone marrow (Fig. 3D).

In the femurs of mature frogs (3 cm in size), the distribution of hematopoietic cells was more strictly localized to a region adjacent to the cortical bone, and were arranged in a belt-like fashion (Fig. 4A, B). Giemsa
Hematopoiesis in Bone Marrow of Xenopus

staining revealed that these hematopoietic cells consisted mainly of immature cells with a rounded nucleus and pale cytoplasmic area, suggesting that they were granulocyte precursors (Fig. 4D). Few apparent eosinophil lineage cells and erythroblasts were observed (Fig. 4D). An eosinophilic osteoid appeared at the boundary between the cartilage and bone marrow at this stage, and hematopoietic foci were infrequently observed adjacent to the osteoid surface (Fig. 4A). At this stage, most of the bone marrow space was occupied by mature adipocytes (Fig. 4B, C), and a small number of similar hematopoietic cells were observed among adipocytes adjacent to cortical bone (Fig. 4E). At the center of the bone marrow, hematopoietic cells were observed surrounding a small number of blood vessels (Fig. 4B, C).

3. Distribution of hematopoietic cells during bone regeneration in X. laevis

Our findings on the distribution of hematopoietic cells in the bone marrow of femurs in X. laevis suggested a close relationship between hematopoiesis and the bone surface. To validate this observation, a supplementary investigation was carried out to determine whether the distribution of hematopoietic cells was associated with newly formed bone during bone regeneration in the diaphyseal region of the femur in X. laevis.

A small round hole was created at the diaphysis of the femurs and bone regeneration observed at 4 and 6 weeks after injury. Histological observation revealed newly-formed bone at the regenerating sites 4 weeks after injury (Fig. 5A). Regeneration of cortical bone showed progression at 6 weeks after injury (Fig. 5B). Importantly, hematopoietic cells appearing on the newly formed bone surface were arranged in a belt-like fashion at the injury site during bone regeneration (arrows in Fig. 5). These results indicate that hematopoiesis occurs on the bone surface, even during the bone regeneration process in X. laevis.

Discussion

Phylogenetically, the bone marrow cavity first appears in amphibians. The other vertebrates, including reptiles, birds, and mammals, have a well-developed bone marrow cavity. In reptiles, birds, and mammals, hematopoiesis occurs mainly in the bone marrow, although other organs also participate physiologically through extramedullary hematopoiesis. This does not occur in humans, however. In amphibians, hematopoiesis differs among species. Further, hematopoiesis in bone marrow differs depending on species in anurans. As demonstrated in this study, bone marrow retained hematopoietic cells in the primitive frog X. laevis, but its dis-
tribution was mainly restricted to regions adjacent to bone. The bone marrow cavity was occupied by adipose tissue from the early developmental stages (Fig. 2F). Tanaka\textsuperscript{26} reported similar findings on the distribution of hematopoietic cells in the bone marrow of \textit{X. laevis}. Others also demonstrated that hematopoietic cells were more abundant in the bone marrow of \textit{Rana catesbeiana} than that of \textit{X. laevis}, although those cells showed preferential distribution on endosteal surfaces\textsuperscript{1,26}.

In other terrestrial frogs, including \textit{Rana rugosa}, \textit{Rana nigromaculata}, and \textit{Rana porosa}, hematopoietic cells were observed irregularly scattered in the bone marrow, including on the endosteal surfaces (unpublished results). This suggests that hematopoiesis in the bone marrow of anurans is weak, and that it differs among species in which it is also observed on the endosteal surface. Similarly, Tanaka\textsuperscript{26} reported an absence of hematopoiesis in the bone marrow of \textit{Triturus pyrrhogaster}, suggesting an absence of hematopoiesis in the bone marrow of urodeles. These observations suggest that the primitive vertebrate \textit{X. laevis} phylogenetically acquired hematopoiesis in bone marrow.

As previously mentioned, hematopoietic stem cells in mammals have been proposed to be retained by two kinds of niche: osteoblastic and vascular\textsuperscript{1,4,19,29}. The results of the present study revealed preferential distribution of hematopoietic cells on the endosteal surfaces of cortical bone in \textit{X. laevis}. In addition, hematopoiesis was observed to occur on the surface of newly-formed bones during bone regeneration in adult \textit{X. laevis}. These results suggest that the bone marrow of \textit{X. laevis} retains an osteoblastic niche. Further investigations are required to confirm the role of the osteoblast niche in \textit{X. laevis}, however, as the role of osteoblasts in the hematopoietic niche remains controversial\textsuperscript{7,8,19,23}. An abundant supply of blood vessels into the bone is necessary in the process of vascular niche construction. Although several blood vessels were observed in the bone marrow of \textit{X. laevis} in the present study, the number of blood vessels associated with hematopoietic cells around these vessels was very small. These findings suggest that the vascular niche is less developed in the bone marrow of \textit{X. laevis} than that in mice. Tanaka\textsuperscript{26} investigated the distribution of blood vessels in the bone marrow of \textit{X. laevis} and \textit{Rana catesbeiana} by injection of Indian ink and showed a venous vascular net in the endosteal region of cortical bone, where hematopoietic cells were observed in the bone marrow. These findings suggest that the vascular niche plays an important role in the maintenance of these hematopoietic cells. Further investigation into the relationship between vascular networks and the distribution of hematopoietic cells in the bone marrow of \textit{X. laevis} is ongoing at our laboratory.

Hematopoiesis occurs in various tissues in adult \textit{X. laevis} such as liver, bone marrow, and kidney, with the liver being the main hematopoietic organ and the bone marrow being a rudimentary tissue\textsuperscript{12}. Cumano and Godin\textsuperscript{4} reported that the kidney, spleen, and thymus were the definitive hematopoietic sites in adult \textit{X. laevis}. Hadji-Azimi\textsuperscript{12} et al. identified bone marrow as the main site of neutrophil differentiation in adult \textit{X. laevis}, whereas eosinophils, erythrocytes, thrombocytes, and monocytes mainly differentiated in the liver. However, Yaparla\textsuperscript{28} et al. reported that committed macrophage precursors resided in the bone marrow and were absent from the liver. Thus, the distribution of hematopoietic cells in various types of tissue in \textit{X. laevis} remains contentious. Various antibodies recognizing surface markers of hematopoietic cells in mammals are often used to confirm the details of cell lineages, but few are available to recognize hematopoietic cells in amphibians, including in \textit{X. laevis}\textsuperscript{1}. Further studies are necessary to determine the cell types involved in hematopoiesis in \textit{X. laevis}, using available antibodies to identify the different cell lineages.

In conclusion, this study investigated the distribution of femoral hematopoietic cells in \textit{X. laevis} at various stages of its development. The results revealed that the preferential distribution of hematopoietic cells on the endos-
teal surfaces was a feature in femurs from *X. laevis* that distinguishes it from that observed in mice.

**Acknowledgements**

This study was supported by a grant of Grant-in-Aid for Challenging Exploratory Research (JSPS KAKENHI 26670657 to AY) and grants for Private University Branding Project supported by Ministry of Education, Culture, Sports, Science and Technology, Japan, and Tokyo Dental College Branding Project for Multidisciplinary Research Center for Jaw Disease (MRCJD): Achieving Longevity and Sustainability by Comprehensive Reconstruction of Oral and Maxillofacial functions.

**Conflict of Interest**

The authors declare no conflict of interest.

**References**

1) Abreu Manso PP, de Brito-Güirana L, Pelajo-Machado M (2009) Localization of hematopoietic cells in the bullfrog (*Lithobates catesbeianus*). Cell Tissue Res 337:301–312.

2) Acar M, Kocherlakota KS, Murphy MM, Peyer JG, Oguro H, Inra CN, Jaiveola C, Zhao Z, Luby-Phelps K, Morrison SJ (2015) Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. Nature 526:126–130.

3) Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, Ito K, Koh GY, Suda T (2004) Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 118:149–161.

4) Brunst VV (1958) The effect of total-body x-irradiation on the adult axolotl (*Siredon mexicanum*). Radiat Res 8:32–45.

5) Campbell FR (1970) Ultrastructure of the bone marrow of the frog. Am J Anat 129:329–355.

6) Chen XD, Turpen JB (1995) Intraembryonic origin of hepatic hematopoiesis in *Xenopus laevis*. J Immunol 154:2557–2567.

7) Ciau-Uitz A, Monteiro R, Kirmizitas A, Patient R (2014) Development hematopoiesis: ontogenesis, genetic programming and conservation. Exp Hematol 42:669–683.

8) Crane GM, Jeffery E, Morrison SJ (2017) Adult haematopoietic stem cell niches. Nat Rev Immunol 9:573–590.

9) Cumano A, Godin I (2007) Ontogeny of the hematopoietic system. Annu Rev Immunol 25:745–785.

10) Dahrowski Z, Sano Martins IS, Tabarowski Z, Witkowska-Pelc E, Spadaccì Morena DD, Spodaryk K, Podkowa D (2007) Haematopoiesis in snakes (*Ophidia*) in early postnatal development. Cell Tissue Res 328:291–299.

11) Gordin I, Cumano A (2005) Of birds and mice: hematopoietic stem cell development. Int J Dev Biol 49:251–257.

12) Hadji-Azimi I, Coosemans V, Canicatti C (1987) Atlas of adult *Xenopus laevis* laevis hematology. Dev Comp Immunol 11:807–874.

13) Imanirad P, Dzierzak E (2013) Hypoxia and HIFs in regulating the development of the hematopoietic system. Blood Cells Mol Dis 51:256–263.

14) Kopp HG, Avevilla ST, Hooper AT, Rafii S (2005) The bone marrow vascular niche: home of HSC differentiation and mobilization. Physiology (Bethesda) 20:349–356.

15) Lopez D, Lin L, Monaghan JR, Cogle CR, Bova FJ, Maden M, Scott EW (2014) Mapping hematopoiesis in a fully regenerative vertebrate: the axolotl. Blood 124:1232–1241.

16) Maeno M (2003) Regulatory signals and tissue interactions in the early hematopoietic cell differentiation in *Xenopus laevis* embryo. Zoolog Sci 20:939–946.

17) Maniatis GM, Ingram VM (1971) Erythropoiesis during amphibian metamorphosis: I. Site of maturation of erythrocytes in Rana catesbeiana. J Cell Biol 49:372–379.

18) Morishita T, Shibata Y, Tsukazaki T, Yamaguchi A (2005) Expression profile of *Xenopus* banded hedgehog, a homolog of mouse Indian hedgehog, is related to the late development of endochondral ossification in *Xenopus laevis*. Biochem Biophys Res Commun 328:867–873.

19) Morrison SJ, Scadden DT (2014) The bone marrow niche for haematopoietic stem cells. Nature 505:327–334.

20) Nieuwkoop PD, Faber J (1956) Normal Table of *Xenopus laevis* (Daudin), Elsevier/North-Holland, New York.

21) Shahrami S, Rezaeeyan H, Ahmadzadeh A, Shahjahani M, Saki N (2016) Bone marrow
blood vessels: Normal and neoplastic niche. Oncol Rev 10:306.

22) Shiozawa Y, Taichman RS (2012) Getting blood from bone: an emerging understanding of the role that osteoblasts play in regulating hematopoietic stem cells within their niche. Exp Hematol 40:685–694.

23) Sugiyama T, Omatsu Y, Nagasawa T (2019) Niches for hematopoietic stem cells and immune cell progenitors. Int Immunol 31: 5–11.

24) Suttie AW (2006) Histopathology of the spleen. Toxicol Pathol 34:466–503.

25) Taichman RS (2005) Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. Blood 105: 2631–2639.

26) Tanaka Y (1976) Architecture of the marrow vasculature in three amphibian species and its significance in hematopoietic development. Am J Anat 145:485–497.

27) Yamamoto K, Miwa Y, Abe-Suzuki S, Abe S, Kirimura S, Onishi I, Kitagawa M, Kurata M (2016) Extramedullary hematopoiesis: Elucidating the function of the hematopoietic stem cell niche. Mol Med Rep 13:587–591.

28) Yaparla A, Wendel ES, Grayfer L (2016) The unique myelopoiesis strategy of the amphibian *Xenopus laevis*. Dev Comp Immunol 63: 136–143.

29) Yin T, Li L (2006) The stem cell niches in bone. J Clin Invest 116:1195–1201.

30) Zhou B, Yu H, Yue R, Zhao Z, Rios JJ, Naveiras O, Morrison SJ (2017) Bone marrow adipocytes promote the regeneration of stem cells and haematopoiesis by secreting SCF. Nat Cell Biol 19:891–903.

Correspondence:

Dr. Akira Yamaguchi
Oral Health Science Center,
Tokyo Dental College,
2-9-18 Kanda-Misakicho, Chiyoda-ku,
Tokyo 101-0061, Japan
E-mail: akira@tdc.ac.jp