Isolation and characterization of hematopoietic stem cells in teleost fish

Isolation and characterization of hematopoietic stem cells in teleost fish

Isao Kobayashi[1)], Fumihiko Katakura[2)], and *Tadaaki Moritomo[2)]

[1)]Faculty of Natural System, Institute of Science and Engineering, Kanazawa University
Kakumamachi, Kanazawa, Ishikawa 920-1192, Japan

[2)]Laboratory of Comparative Immunology, Department of Veterinary Medicine, Nihon University
Kameino 1866, Fujisawa, Kanagawa 252-0880, Japan

*Corresponding author
Email: moritomo.tadaaki@nihon-u.ac.jp
Tel/FAX: +81-0466-84-3374

Abstract

Despite 400 million years of evolutionary divergence, hematopoiesis is highly conserved between mammals and teleost fish. All types of mature blood cells including the erythroid, myeloid, and lymphoid lineages show a high degree of similarity to their mammalian counterparts at the morphological and molecular level. Hematopoietic stem cells (HSCs) are cells that are capable of self-renewal and differentiating into all hematopoietic lineages over the lifetime of an organism. The study of HSCs has been facilitated through bone marrow transplantation experiments developed in the mouse model. In the last decade, the zebrafish and clonal ginbuna carp (*Carassius auratus langsdorfii*) have emerged as new models for the study of HSCs. This review highlights the recent progress and future prospects of studying HSCs in teleost fish. Transplantation assays using these teleost models have demonstrated the presence of HSCs in the kidney, which is the major hematopoietic organ in teleost fish. Moreover, it is possible to purify HSCs from the kidney utilizing fluorescent dyes or transgenic animals. These teleost models will provide novel insights into the universal mechanisms of HSC maintenance, homeostasis, and differentiation among vertebrates.

Keywords

Hematopoietic stem cell, teleost, ginbuna carp, zebrafish, transplantation, stem cell niche

Highlights

- Transplantation model systems were established in the zebrafish and clonal ginbuna carp to evaluate the activity of hematopoietic cells.

- Hematopoietic stem cells are present in the kidney, but not in the spleen in teleost fish.

- The frequency of hematopoietic stem cells in the zebrafish kidney is similar to the murine bone marrow.

- Hematopoietic stem cells can be purified using fluorescent dyes or transgenic animals in teleost models.

- Kidney renal tissues (e.g. renal tubules) may play an important role in the maintenance of hematopoietic stem cells as the niche.

1. Introduction

Hematopoietic stem cells (HSCs) are rare cells with the remarkable ability to both self-renew and generate all mature blood cell types over the lifetime of an individual (Orkin & Zon, 2008). HSCs are used therapeutically in the treatment of numerous diseases including leukemia and congenital blood disorders, but obtaining suitable cells for transplantation remains a problem. HSCs present within adult bone marrow or newborn cord blood are by far the most widely utilized human stem cells in the clinic. It is often difficult, however, to obtain sufficient numbers of HSCs for adult transplantation. Despite three decades of efforts, it has not yet been possible to maintain or expand HSCs *ex vivo* for regenerative medicine approaches. A major obstacle has been a lack of understanding of the key cell types and signaling pathways that comprise the HSC niche, which is defined as the cellular and molecular components that regulate HSC quiescence, self-renewal, and differentiation (Li & Xie, 2005; Moore & Lemischka, 2006; Morrison & Spradling, 2008; Ema & Suda, 2012).

In adult teleosts, hematopoiesis is maintained in the kidney, where all lineages of hematopoietic differentiation are observed including erythropoiesis, myelopoiesis, and lymphopoiesis. Thus, the teleost kidney is thought to be equivalent to the mammalian bone marrow (Zapata, 1979; 1981). Although the teleost kidney is of great importance to understand HSC niches and the process of hematopoietic differentiation, until recently, little information has been available regarding HSCs nor their niches in the kidney. Considerable knowledge of HSCs in the mammalian bone marrow has been obtained through *in vivo* experiments. The transplantation of donor hematopoietic cells into the histocompatible recipients provides a system of assaying the differentiation and repopulating capacity of the hematopoietic cells (Morrison et al., 1995). In the 2000s, similar assay systems of HSCs were developed in the zebrafish and ginbuna carp (*Carassius auratus langsdorfii*). In this review, we discuss the *in vivo* assay systems and purification strategies of HSCs in teleost fish. In addition, we also discuss our understanding of HSC niches in the teleost kidney.

2.1. Methods for assaying HSCs in teleost fish

2.1.1. Transplantation systems in the zebrafish and ginbuna carp

The study of hematopoiesis in teleosts began with the identification of the primary hematopoietic organs through morphological analysis. Various stages of hematopoietic cells were observed in the interstitial tissue of the carp kidney by electron microscopy (Zapata, 1979; 1981). This hematopoietic site in the kidney was later termed the

"kidney marrow" in zebrafish (Traver et al., 2003). Although it was predicted that HSCs would also be present in the kidney, HSCs could not be distinguished morphologically from other hematopoietic cells, and at that time, there was no direct functional evidence for the presence of HSCs in the teleost kidney. In mammalian systems, the only valid test for defining HSCs is the demonstration of their capacity for long-term and multi-lineage repopulation of the hematopoietic system following transplantation (Wognum et al., 2003). Pioneering methods for the transplantation of hematopoietic cells in zebrafish were first reported by L. I. Zon's group, enabling the assay of reconstitutive potential in teleosts (Traver et al., 2003). The Zon group utilized transgenic zebrafish that express fluorescent proteins in different cell types, including *gata1:DsRed* (which is expressed in erythroid lineage) and *bactin:GFP* (which is expressed in all blood cell lineages except mature erythrocytes). Due to the lack of inbred strains in zebrafish, the Zon group employed pre-thymic stage embryos as recipients to avoid allogeneic transplant rejection. After injection of fluorescent protein-labeled kidney hematopoietic cells ("whole kidney marrow cells") into recipient animals, donor-derived erythroid and myeloid cells were detected in the recipient kidney and blood circulation. Using this approach, they determined that cells from the "lymphoid" fraction, which was identified as a cell population with low levels of forward and side scatter (FS^{low} SS^{low}) by flow cytometry, possess long-term (> 6 months) hematopoietic repopulation capacity (Traver et al., 2003). This was the first evidence for the presence of HSCs in the teleost kidney. However, successful long-term engraftment of donor cells was observed in only 2 out of 110 recipients, highlighting the difficulty of this transplantation assay in the zebrafish model.

Our group also has developed a method for assaying hematopoietic cells using clonal ginbuna carps and ginbuna-goldfish (*Carassius auratus*) hybrids (Moritomo et al., 2004). Clonal ginbuna carps are unisex triploid fish (all female, 3n = 156) that principally reproduce gynogenetically in nature. One particular clone isolated from Lake Suwa, Japan (S3n strain) has been especially useful in our studies. This clone is unique in that it can be reproduced by not only gynogenesis but also bisexual reproduction. When eggs from the S3n clone are inseminated with normal goldfish sperm, tetraploid hybrids (S4n) are obtained. In contrast, when eggs are inseminated with goldfish sperm inactivated by UV irradiation, triploid clones are obtained (Fig. 1). The S4n fish possess four sets of chromosomes, three from the S3n clone and one from the goldfish. Therefore, when cells obtained from S3n clones are transferred into S4n recipients, transplants are accepted, whereas vice versa transplants are rejected (Nakanishi & Ototake, 1999). Moreover, since all blood cells, including erythrocytes and

thrombocytes (equivalent to mammalian platelets), possess a nucleus in teleosts, donor and recipient cells can be easily distinguished through ploidy analysis using flow cytometry (Fischer et al., 1998). Thus, the transplantation of S3n donor cells into S4n recipients allows us to trace all types of donor-derived blood cells for a long period and is suitable for the assay of HSCs.

We demonstrated the presence of HSCs in the ginbuna carp kidney using the "S3n donor and S4n recipient" system (Kobayashi et al., 2006). A cell fraction enriched with hematopoietic cells was obtained from the S3n kidney based on a density gradient to exclude mature erythrocytes and granulocytes and injected into S4n recipients that were induced with severe anemia in order to promote the engraftment of donor hematopoietic cells. After transplantation, blood samples were collected from the recipients, and the percentage of donor-derived cells was measured by ploidy analysis. Fig. 2a and b show a typical ploidy analysis of erythrocytes and leukocytes, respectively. Donor-derived triploid blood cells began to appear 3 months after transplantation, and more than half of the cells were donor origin in some recipients at 12 months post-transplantation (mpt). The multi-lineage contribution of donor cells was examined in the recipient animals at 12 mpt. Donor-derived leukocytes (S3n) sorted from S4n recipients were morphologically examined. All major types of leukocytes were found in the recipients, including neutrophils, basophils, monocytes, thrombocytes and lymphocytes (Fig. 2c). In addition, expression of the T and B lymphocyte marker genes, T cell receptor ß (*TCR-ß*) and immunoglobulin light-chain (*IgL*), was also detected in donor-derived leukocytes. Although the transplanted cells include multiple immature progenitors, only HSCs can contribute to the sustained, long-term production of blood cells (> 6 months) (Osawa et al., 1996; Zhong et al., 1996). Taken together, these results indicate that HSCs, characterized by their capacity for long-term and multi-lineage repopulation, are present in the ginbuna carp kidney (Kobayashi et al., 2006).

While the "S3n donor and S4n recipient" transplantation system has some advantages to test the activity of HSCs, there remains a problem in this system. When S3n cells were transferred into S4n recipients, most of the S4n recipients died with graft-versus-host disease (GVHD)-like symptoms between 6 - 18 mpt (Kobayashi et al., 2006). The onset of illness appeared to be a loss of appetite followed by scale protrusion, severe hemorrhage, and local destruction of the ventral skin, similar to GVHD symptoms previously reported in ginbuna carps (Nakanishi & Ototake, 1999). In this transplantation system, S4n recipients are genetically tolerant of S3n donor cells, but transplanted S3n-derived T cells are able to react against allogeneic antigens of the S4n recipient. In fact, Fischer et al. reported that transplantation of leukocytes from S3n

donors that were immunized with recipient-derived S4n cells led to the acute GVHD in the recipients and death within 1 month (Fischer et al., 2006). In our studies, donor fish were not pre-sensitized with S4n cells, allowing the recipients to survive longer without developing acute GVHD. However, due to their prolonged presence in the host, donor-derived leukocytes may eventually mount specific immune responses that result in the observed chronic GVHD and death of the S4n recipients.

2.1.2. The use of irradiated recipients for transplantation assays

Most strategies for transplantation have involved cytoablative treatments, usually total body irradiation. This is based on the assumption that there are specific marrow areas or niches to which engrafting HSCs home. If they are occupied by hostresident HSCs, HSC engraftments do not take place (Schofield, 1978). Since, teleosts are generally more resistant to radiation than mammals, an important step in developing teleost transplantation assays was determining effective cytoablative doses of irradiation. In goldfish, the minimum lethal dose (MLD), which is defined as a dose that causes more than 90% of mortality, is approximately 30 Gy (Shechmeister et al., 1962), whereas that of mice is 9 Gy (Congdon et al., 1952). Traver et al. reported that the MLD of zebrafish was approximately 40 Gy, and that hematopoiesis never recovered in 40 Gy-irradiated fish, indicating that nearly all hematopoietic subsets, including HSCs, are depleted at this dose. Transplantation of kidney hematopoietic cells from normal donors rescued the 40 Gy-irradiated recipients (Traver et al., 2004). Thus, transplantation of hematopoietic cells from the teleost kidney recapitulates the radioprotective effect of comparable transplants in the mouse model (Lorenz et al., 1951). This confirmation allows us to evaluate the activity of hematopoietic stem/progenitor cells (HSPCs) based on the radioprotective activity of donor cells. Langenau et al. examined the recovery of the thymus in irradiated recipients by transplantation of hematopoietic cells from *lck:GFP* fish, in which T lymphocytes are specifically labeled with GFP. Transplantation of thymocytes and kidney marrow cells from *lck:GFP* animals resulted in the transient and permanent repopulation of the thymus in the recipients, respectively. These results indicate that the thymus contains only short-term thymic repopulating progenitors, whereas long-term repopulating HSCs are present in the kidney (Langenau et al., 2004).

We have also developed a transplantation system using X-ray-irradiated ginbuna carps. The MLD of ginbuna carps was approximately 25 Gy, which was strong enough to deplete all hematopoietic cells in the ginbuna carp. To avoid the transplant rejection and GVHD, we utilized the same stain (OB1 stain) for both donors and recipients.

Whole leukocytes (exclude mature erythrocytes) from the head kidney (HK), trunk kidney (TK), or spleen were separately transferred into 25 Gy-irradiated recipients, and the survival rate of these recipients was examined. Approximately 50 and 77% of the recipients of HK and TK cells survived more than 180 days post irradiation (dpi), respectively, whereas all of the recipients transferred with spleen cells died within 30 dpi. These results clearly indicate that HSCs reside in the HK and TK, but not in the spleen of ginbuna carps (Kobayashi et al., 2008a). It has been reported that the spleen from adult mice contains HSCs capable of long-term and multi-lineage reconstitution in lethally irradiated hosts, while the number of HSCs in the spleen is smaller than that in the bone marrow (Wolber et al., 2002; Morita et al., 2011). The spleen in teleosts is also thought to be involved in erythropoiesis in adult fish, although its role in this process seems to be secondary to the kidney (Davies et al., 1975). Several groups have suggested through morphological analysis that the function of the teleost spleen is erythroclasis (fragmentation of erythrocytes) rather than erythropoiesis (Al-Adhami et al., 1976; Quesada et al., 2005). Taken together, these results suggest that the role of the teleost spleen in hematopoiesis is more limited compared to the mammalian spleen, and that erythropoiesis in teleosts occurs in the kidney rather than spleen.

2.1.3. Improved methods of zebrafish transplantation

Recent studies have demonstrated improved methods of transplantation in zebrafish using major histocompatibility complex (MHC)-matched or immune-deficient recipients (de Jong et al., 2011; Hess et al., 2013; Tang et al., 2014). de Jong et al. defined MHC haplotypes in zebrafish, and found that MHC-matched recipients show dramatically increased engraftment and chimerism when compared with unmatched recipients (de Jong et al., 2011). Hess et al. established an elegant transplantation system using *cmyb* mutants in which a point mutation in *cmyb* results in the complete loss of functional HSCs from the embryonic stage. These mutants, however, can survive for several weeks because embryonic primitive hematopoiesis is unaffected by the *cmyb* mutation (Soza-Ried et al., 2010). Injection of kidney hematopoietic cells into 1-month-old *cmyb* mutants successfully rescued hematopoiesis in these mutants without cytoablative treatments (Hess et al., 2013). Tang et al. utilized *rag2* mutant zebrafish as a recipient that lacks both T and B lymphocytes, but survives to adulthood. Sublethally irradiated *rag2* mutants exhibited robust and multi-lineage blood cell engraftment without rejection by host cells, whereas none of wild type recipients exhibited engraftment at the same condition (Tang et al., 2015). Based on limiting dilution assays using MHC-matched and *cmyb* mutant recipients, the frequency of

HSCs in the zebrafish kidney was estimated at 1 in 65,500 (de Jong et al., 2011) and 1 in 38,140 cells (Hess et al., 2013), respectively. Although variable, these data are comparable to the frequency of HSCs found in the murine bone marrow (approximately 1 in 30,000) (Bryder et al., 2006).

2.2. Isolation of HSCs from the teleost kidney

2.2.1. Fluorescent dye-based HSC purification in teleost fish

The prospective isolation of HSCs is essential for the molecular and functional analysis of HSCs. Purification of HSCs in mammals has been traditionally achieved by flow cytometry using monoclonal antibodies (mAbs) (Wognum et al., 2003). Although CD34 has been commonly used as a marker for HSCs in mammals (Berenson et al., 1988; Spangrude and Johnson, 1990; Bruno et al., 1999; Verfaillie et al., 1990), murine HSCs have been recognized as CD34-negative (Osawa et al., 1996; Goodell et al., 1997). Instead of CD34, stem cell antigen-1 (Sca-1) and c-kit are generally used for identification of murine HSCs (Osawa et al., 1996; Spangrude et al., 1988). In addition, murine HSCs can be distinguished by the expression of SLAM family receptors (CD150 and CD48) (Kiel et al., 2005). Thus, the expression of surface markers in HSCs differs between species. On the other hand, HSCs in the bone marrow are also identified on the basis of the efflux activity of fluorescent dyes, such as the DNA-binding dye Hoechst 33342 (Hoechst) and the mitochondrial dye Rhodamine-123 (Rho) (Wolf et al., 1993; Leemhuis et al., 1996; Goodell et al., 1996; 1997). The Hoechst efflux activity of HSCs is attributed to the expression of an ATP binding cassette (ABC) transporter, ABCG2 (Zhou et al., 2001; 2002). Hoechst-low HSCs can be identified as side population (SP) cells by flow cytometry (Goodell et al., 1996; 1997). In contrast, the Rho efflux activity of HSCs is attributed to another ABC transporter, P-glycoprotein (P-gp) (Uchida et al., 2002), and the Rho-negative (Rho^{-}) population is also enriched for HSCs in the bone marrow (Spangrude and Johnson, 1990; Orlic et al., 1993). Importantly, the dye efflux activities of HSCs are widely conserved in mammals (Goodell et al., 1997; Baum et al., 1992; Mahmud et al., 2001), suggesting that fluorescent dye-based purification strategies could potentially be used as a universal method of HSC isolation in vertebrates. We and others attempted to isolate SP cells from the teleost kidney (Kobayashi et al., 2007; Tsinkalovsky et al., 2007; Kobayashi et al., 2008b). Tsinkalovsky et al. identified a putative SP fraction in the zebrafish kidney. The percentage of the SP fraction in the zebrafish kidney was approximately 0.3%, which is similar with that in the murine bone marrow (approximately 0.4%) (Matsuzaki et al.,

2004). This fraction was reduced by treatment with reserpine, an inhibitor of ABC transporters. In addition, cells in the SP fraction highly expressed some HSC-related genes, such as *scl*, *lmo2*, *cmyb*, and *tie2* when compared with the non-SP fraction (Tsinkalovsky et al., 2007). The SP fraction shown by Tsinkalovsky et al., however, was detected as a small cluster completely separated from the non-SP fraction. In general, SP cells exhibit a gradient of efflux activity and are identified as a small streak extending from the non-SP fraction. In addition, the SP cells identified by Tsinkalovsky et al. were 3μm in diameter, which is much smaller than normal zebrafish hematopoietic cells (for example, zebrafish lymphocytes are 5 - 7μm in diameter) (Traver et al., 2003; Kobayashi et al., 2008b). Therefore, the population identified by Tsinkalovsky et al. might contain non-hematopoietic cells and might not represent a characteristic SP fraction.

We have also identified SP cells in the ginbuna carp kidney (Kobayashi et al., 2007). Whole leukocytes from the ginbuna carp kidney were stained with Hoechst and analyzed by flow cytometry. Fig. 3 shows a typical flow cytometric pattern of kidney hematopoietic cells stained with Hoechst. The Hoechst-low SP fraction was observed as a small streak population in the ginbuna carp kidney. The frequency of SP cells was approximately 0.17% in the whole kidney, and this fraction was nearly undetectable when hematopoietic cells were treated with verapamil, an inhibitor of ABC transporters. Cells in the SP fraction were morphologically uniform, composed of small lymphocyte-like cells having a round nucleus with a thin-layered cytoplasm. These characteristics are very similar to the SP fraction found in the mouse (Goodell et al., 1996; 1997). The HSC activity of SP cells was evaluated by the transplantation using the "S3n donor and S4n recipient" system. After injection of SP cells from S3n donors into S4n recipients, ploidy analysis was performed in the recipients. Donor-derived cells were first detected 4 mpt, and they were sustained for more than 9 to 16 mpt. These donor-derived cells included multiple types of blood cells, such as erythrocytes, neutrophils, basophils, monocytes, lymphocytes and thrombocytes. In addition, the expression of T and B lymphocyte markers was also detected from the donor-derived cells, confirming that SP cells in the ginbuna carp kidney include HSCs (Kobayashi et al., 2007).

Kidney SP cells were also identified in the zebrafish (Kobayashi et al., 2008b; 2010) and medaka (*Oryzias latipes*) (Fig. 4). The percentage of kidney SP cells in the zebrafish and medaka was approximately 0.096 and 0.18%, respectively. Although the flow cytometric pattern of SP cells in the zebrafish and medaka was very similar to that in the ginbuna carp, cells in the SP fraction were heterogenous in the zebrafish kidney.

More than 50% of zebrafish SP cells fell into the FS^{low} SS^{low} lymphoid fraction, whereas the remaining cells were in the FS^{high} myeloid fraction, which includes mature and immature granulocytes (Kobayashi et al., 2008b). This suggests that the "SP phenotype" is variable in species. Importantly, however, the molecular mechanism of the Hoechst dye efflux activity in SP cells is conserved among vertebrates, as evidenced by the conserved expression and function of Abcg2 in murine and zebrafish SP cells (Zhou et al., 2001; Kobayashi et al., 2008b). Based on comparative microarray analysis, we found that the molecular signature of zebrafish "lymphoid SP" cells is similar to murine and human HSCs. Genes involved in cell adhesion and signal transduction tended to be upregulated in these HSC fractions, whereas genes involved in DNA replication were generally downregulated. This expression pattern reflects that HSCs adhere to their niche cells and express some signaling molecules, but are in a quiescent state of the cell cycle. Moreover, these HSC fractions from the zebrafish, mouse, and human shared essential transcription factors related with HSC function, such as *Gata2*, *Id1*, and *Egr1*. Thus, lymphoid SP cells in the zebrafish kidney possess typical features of HSCs (Kobayashi et al., 2010).

The Rho efflux activity of HSCs is also conserved in ginbuna carps (Kobayashi et al., 2008c). Rho^- cells were detected in the ginbuna carp kidney a frequency of approximately 0.64%. These cells were mainly composed of two types of cells, small round lymphoid cells and relatively cytoplasm-abundant blast cells. The Rho^- fraction was reduced by treatment with reserpine, suggesting that the low level of fluorescence in Rho^- cells is mediated by ABC transporters. The Rho^- fraction was partially merged with the SP fraction, as in the murine bone marrow (Hüttmann et al., 2001; Uchida et al., 2004). The Rho^- SP fraction was approximately 0.097% in the ginbuna carp kidney, while the equivalent fraction in the murine bone marrow was approximately 0.01% (Uchida et al., 2004). Staining with an mAb cocktail against lymphocytes in ginbuna carps (CD4, CD8α, and IgM) revealed that approximately 42.6% of Rho^- cells expressed markers of mature lymphocytes, while these markers were never detected in SP cells. Transplantation of Rho^- cells from S3n donors into S4n recipients resulted in long-term hematopoietic repopulation by donor-derived cells, indicating that HSCs are also present in the Rho^- fraction of the ginbuna carp kidney (Kobayashi et al., 2008c). Although functionally untested, Rho^- SP cells isolated from the teleost kidney may represent a further purified population of HSCs as has been demonstrated in the mouse (Hüttmann et al., 2001; Uchida et al., 2004).

2.2.1. Isolation of HSCs using transgenic zebrafish

Recently, HSC fractions have been identified in the zebrafish kidney using transgenic animals including the *cd41:GFP* (Ma et al., 2011) and *Runx1:mCherry* (Tamplin et al., 2015). The *cd41:GFP* zebrafish was initially generated to trace developing thrombocytes in the embryonic stage (Lin et al., 2005). Later, this line was used for the imaging of HSPCs that emerge from the dorsal aorta in the zebrafish embryo (Murayama et al., 2006; Kissa et al., 2008; Bertrand et al., 2008). Ma et al. identified the two different populations of *cd41:GFP*$^{+}$ cells in the adult kidney, *cd41:GFP*^{high} and *cd41:GFP*^{low}. Based on transplantation experiments, they found that cells in the *cd41:GFP*^{low} fraction have the capacity for long-term and multi-lineage repopulation, whereas cells in the *cd41:GFP*^{high} fraction do not have this potential (Ma et al., 2011). Tamplin et al. recently established transgenic zebrafish that express GFP or mCherry under the control of a regulatory element from the first intron of the mouse *Runx1* locus (*Runx1:GFP or Runx1:mCherry*). Both the *Runx1:GFP* and *Runx1:mCherry* mark zebrafish HSPC in the embryo and the adult kidney. The HSC activity of *Runx1:mCherry*$^{+}$ cells has been confirmed by long-term transplantation experiments (Tamplin et al., 2015). Limiting dilution assays revealed that the frequency of HSCs in the *cd41:GFP*^{low} and *Runx1:mCherry*$^{+}$ fraction was approximately 1 in 100 (Ma et al., 2011) and 1 in 35 (Tamplin et al., 2015), respectively. Lineage-tracing of HSCs using the Cre/loxP system has also been developed in the zebrafish. In order to trace nascent HSCs in the zebrafish embryo, Bertrand et al. combined the *bactin2:loxP-STOP-loxP-DsRed* reporter animal with a *kdrl:Cre* line, which specifically expresses Cre in vascular endothelial cells. They found that nearly all adult blood cells were marked by DsRed expression in these double-transgenic animals (Bertrand et al., 2010), indicating that all adult HSCs derived from hemogenic endothelium, a specialized subset of endothelium that can differentiate into HSPCs in the embryonic stage (Swiers et al., 2013). Similar results were obtained using *jam1a:CreER*^{T2} (Kobayashi et al., 2014) and *gata2b:Gal4*; *UAS:Cre* animals (Butko et al., 2015). Thus, new valuable tools have been generated to purify and trace HSCs in the zebrafish model.

2.3. The HSC niche in the teleost kidney

The HSC niche was first proposed by Schofield in 1978 as a special microenvironment in which the reconstitutive potential of HSCs is maintained (Schofield, 1978). Recent studies have identified some essential cellular components of the HSC niche in the murine bone marrow, including osteoblasts (Calvi et al., 2003; Zhang et al., 2003; Arai et al., 2004), sinusoidal endothelial cells (Kiel et al., 2005),

mesenchymal stem cells (MSCs) (Mendez-Ferrer et al., 2010; Kunisaki et al., 2013), perivascular cells (Sugiyama et al., 2006), macrophages (Winkler et al., 2010), and megakaryocytes (Zhao et al., 2014; Bruns et al., 2014; Nakamura-Ishizu et al., 2014). These niche cells express a high level of HSC maintenance factors termed "niche factors", including Angiopoietin 1 (Ang-1) (Arai et al., 2004), stem cell factor (SCF) (Heissig et al., 2002), CXCL12 (Sugiyama et al., 2006; Katayama et al., 2006; Kollet et al., 2006), thrombopoietin (Tpo) (Qian et al., 2007; Yoshihara et al., 2007), Wnt (Fleming et al., 2008), Jagged 1 (Jag1) (Calvi et al., 2003; Butler et al., 2010), TGF-β (Yamazaki et al., 2011), osteopontin (OPN) (Nilsson et al., 2005; Stier et al., 2005) CXCL4 (Bruns et al., 2014), N-cadherin (Haug et al., 2008; Hosokawa et al., 2010a; 2010b), and E-selectin (Winkler et al., 2012). There are, however, still controversies in the field regarding the precise HSC niche, and a better understanding of the elements that regulate HSCs is required.

The kidney of cyprinid fish, including ginbuna carps and zebrafish, can be divided into two parts, the HK and TK. The HK has been considered to be a more important hematopoietic organ than the TK, since the HK consists of mostly lympho-hematopoietic tissues, whereas the TK contains abundant urinary tissues. Accordingly, most hematological studies have focused on the HK (Imagawa et al., 1994; Temmink & Bayne, 1988; Bayne, 1986) with the exception of a few studies on the TK (Huttenhuis et al., 2006). We compared the HSCs activity of hematopoietic cells between the HK and TK of ginbuna carps using the "S3n donor and S4n recipient" transplantation system. When HK cells were transferred (n = 3), the chimerism was quite low (only 0 – 3%) except for in one recipient. In contrast, when TK cells were transferred (n = 3), the chimerism was relatively higher (33 – 96%) than that of recipients transferred with HK cells (Kobayashi et al., 2006). Since chimerism reflects the number of HSCs in the donor cells (Harrison, 1980), the TK may contain more HSCs than the HK in ginbuna carps. Consistent with this data, SP cells in ginbuna carps were detected only from the TK, but hardly detected from the HK, spleen, or peripheral blood (Kobayashi et al., 2007). These results suggest that the TK is the major source of HSCs, and that HSC niches are established mainly in the TK in ginbuna carps.

We have determined the distribution of zebrafish *abcg2a*-expressing cells, which are equivalent to SP cells, and found that they are predominantly localized on the basal surface of the renal tubules in the kidney. Similarly, more than 50% of fluorescently labeled SP cells from ginbuna carps home to the basal surface of the renal tubules in the ginbuna carp kidney (Kobayashi et al., 2008b; 2010). Glass et al. generated a transgenic zebrafish that expresses DsRed under the control of *sdf1a* promoter/enhancer elements,

whose protein product has been strongly implicated in the homing of HSCs to the hematopoietic site in mammals (Peled et al., 2000; Sugiyama et al., 2006; Lapidot et al., 2005). They found that *sdf1a:DsRed* is mainly expressed in the renal tubules of zebrafish (Glass et al., 2011). Taken together, these results suggest that renal tubules are a key component of the HSC niche in the teleost kidney. Since the TK contains more renal tubules than the HK, it is likely that HSCs predominantly reside in the TK of ginbuna carps.

3. Future prospects of studying HSCs and HSC niches in the teleost kidney

In the last two decades, the zebrafish has emerged as an exciting animal model system for studying the hematopoietic system (Paw and Zon, 2000; Langenau and Zon, 2005). Most studies in zebrafish, however, have focused on the development of hematopoiesis because of the ease of manipulation and visualization of the zebrafish embryo. While the study of adult hematopoiesis is still limited, a number of unique tools have uncovered fundamental aspects of HSC biology in zebrafish and ginbuna carps. Although the kidney is a very different hematopoietic environment from the mammalian bone marrow, this, in turn, presents an opportunity to discover universal mechanisms of HSC maintenance, homeostasis, and differentiation in vertebrates. As transplantation methods in zebrafish and ginbuna carps are further improved to achieve genuine syngenic transplantation of hematopoietic cells, it should be possible to determine the relative engraftment efficiency between genetically different hematopoietic cell populations. These experiments will advance the use of teleost models in the study of human diseases including hematopoietic malignancies. We believe that the zebrafish and ginbuna carp represent ideal systems in which to dissect the fundamental properties of the HSC niche, and make new discoveries regarding the molecular cues needed to maintain and expand HSCs *ex vivo*.

Acknowledgement

The authors thank K. Lewis for critical evaluation for the manuscript.

References

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

Al-Adhami, M.A., Kunz, Y.W. 1976. Haemopoietic centres in the developing angelfish, *Pterophyllum scalare* (cuvier and valenciennes). Dev. Genes Evol. 176, 393–401.

Baum, C.M.,Weissman, I.L., Tsukamoto, A.S., Buckle, A.M., Peault, B., 1992. Isolation of a candidate human hematopoietic stem-cell population. Proc. Natl. Acad. Sci. U. S. A. 89, 2804–2808.

Bayne, C.J., 1986. Pronephric leucocytes of *Cyprinus carpio*: isolation, separation and characterization. Vet. Immunol. Immunopathol. 12, 141-151.

Berenson, R.J., Andrews, R.G., Bensinger, W.I., Kalamasz, D., Knitter, G., Buckner, C.D., Bernstein, I.D., 1988. Antigen CD34+ marrow cells engraft lethally irradiated baboons. J. Clin. Invest. 81, 951–955.

Bertrand, J.Y., Chi, N.C., Santoso, B., Teng, S., Stainier, D.Y., Traver, D., 2010. Haematopoietic stem cells derive directly from aortic endothelium during development. Nature 464, 108-111.

Bertrand, J.Y., Kim, A.D., Teng, S., Traver, D., 2008. CD41+ cmyb+ precursors colonize the zebrafish pronephros by a novel migration route to initiate adult hematopoiesis. Development 135, 1853-1862.

Bruno, B., Nash, R.A., Wallace, P.M., Gass, M.J., Thompson, J., Storb, R., McSweeney, P.A., 1999. CD34+ selected bone marrow grafts are radioprotective and establish mixed chimerism in dogs given high dose total body irradiation. Transplantation 68, 338–344.

Bruns, I., Lucas, D., Pinho, S., Ahmed, J., Lambert, M.P., Kunisaki, Y., Scheiermann, C., Schiff, L., Poncz, M., Bergman, A., Frenette, P.S., 2014. Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. Nat. Med. 20, 1315-1320.

Bryder, D., Rossi, D.J., Weissman, I.L., 2006. Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. Am. J. Pathol. 169, 338-346.

Butko, E., Distel, M., Pouget, C., Weijts, B., Kobayashi, I., Ng, K., Mosimann, C.,

Poulain, F.E., McPherson, A., Ni, C.W., Stachura, D.L., Del Cid, N., Espin-Palazon, R., Lawson, N.D., Dorsky, R., Clements, W.K., Traver, D., 2015. Gata2b is a restricted early regulator of hemogenic endothelium in the zebrafish embryo. Development 142, 1050-1061.

Butler, J.M., Nolan, D.J., Vertes, E.L., Varnum-Finney, B., Kobayashi, H., Hooper, A.T., Seandel, M., Shido, K., White, I.A., Kobayashi, M., Witte, L., May, C., Shawber, C., Kimura, Y., Kitajewski, J., Rosenwaks, Z., Bernstein, I.D., Rafii, S., 2010. Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. Cell Stem Cell 6, 251-264.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R., Milner, L.A., Kronenberg, H.M., Scadden, D.T., 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425, 841-846.

Congdon, C.C., Uphoff, D., Lorenz, E., 1952. Modification of acute irradiation injury in mice and guinea pigs by injection of bone marrow; a histopathologic study. J. Natl. Cancer Inst. 13, 73-107.

Davies, H.G., Haynes, M.E., 1975. Light- and electron-microscope observations on certain leukocytes in a teleost fish and a comparison of the envelope-limited monolayers of chromatin structural units in different species. J. Cell Sci. 17, 263-285.

de Jong, J.L., Burns, C.E., Chen, A.T., Pugach, E., Mayhall, E.A., Smith, A.C., Feldman, H.A., Zhou, Y., Zon, L.I., 2011. Characterization of immune-matched hematopoietic transplantation in zebrafish. Blood 117, 4234-4242.

Ema, H., Suda, T., 2012. Two anatomically distinct niches regulate stem cell activity. Blood 120, 2174-2181.

Fischer, U., Ototake, M., Nakanishi, T., 1998. 'Life span of circulating blood cells in ginbuna crucian carp (*Carassius auratus langsdorfii*)'. Fish Shellfish Immunol. 8, 339-349.

Fischer, U., Utke, K., Somamoto, T., Köllner, B., Ototake, M., Nakanishi, T., 2006. 'Cytotoxic activities of fish leucocytes'. Fish Shellfish Immunol. 20, 209-226.

Fleming, H.E., Janzen, V., Lo Celso, C., Guo, J., Leahy, K.M., Kronenberg, H.M., Scadden, D.T., 2008. Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal *in vivo*. Cell Stem Cell 2, 274-283.

Glass, T.J., Lund, T.C., Patrinostro, X., Tolar, J., Bowman, T.V., Zon, L.I., Blazar, B.R., 2011. Stromal cell-derived factor-1 and hematopoietic cell homing in an adult zebrafish model of hematopoietic cell transplantation. Blood 118, 766-774.

Goodell, M.A., Brose, K., Paradis, G., Conner, A.S., Mulligan, R.C., 1996. Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. J. Exp. Med. 183, 1797-1806.

Goodell, M.A., Rosenzweig, M., Kim, H., Marks, D.F., DeMaria, M., Paradis, G., Grupp, S.A., Sieff, C.A., Mulligan, R.C., Johnson, R.P., 1997. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat. Med. 3, 1337-1345.

Harrison, D.E., 1980. Competitive repopulation: a new assay for long-term stem cell functional capacity. Blood 55, 77-81.

Haug, J.S., He, X.C., Grindley, J.C., Wunderlich, J.P., Gaudenz, K., Ross, J.T., Paulson, A., Wagner, K.P., Xie, Y., Zhu, R., Yin, T., Perry, J.M., Hembree, M.J., Redenbaugh, E.P., Radice, G.L., Seidel, C., Li, L., 2008. N-cadherin expression level distinguishes reserved versus primed states of hematopoietic stem cells. Cell Stem Cell 2, 367-379.

Heissig, B., Hattori, K., Dias, S., Friedrich, M., Ferris, B., Hackett, N.R., Crystal, R.G., Besmer, P., Lyden, D., Moore, M.A., Werb, Z., Rafii, S., 2002. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 109, 625-637.

Hess, I., Iwanami, N., Schorpp, M., Boehm, T., 2013. Zebrafish model for allogeneic

hematopoietic cell transplantation not requiring preconditioning. Proc. Natl. Acad. Sci. U. S. A. 110, 4327-4332.

Hosokawa, K., Arai, F., Yoshihara, H., Iwasaki, H., Hembree, M., Yin, T., Nakamura, Y., Gomei, Y., Takubo, K., Shiama, H., Matsuoka, S., Li, L., Suda, T., 2010. Cadherin-based adhesion is a potential target for niche manipulation to protect hematopoietic stem cells in adult bone marrow. Cell Stem Cell 6, 194-198.

Hosokawa, K., Arai, F., Yoshihara, H., Iwasaki, H., Nakamura, Y., Gomei, Y., Suda, T., 2010. Knockdown of N-cadherin suppresses the long-term engraftment of hematopoietic stem cells. Blood 116, 554-563.

Huttenhuis, H.B., Taverne-Thiele, A.J., Grou, C.P., Bergsma, J., Saeij, J.P., Nakayasu, C., Rombout, J.H., 2006. Ontogeny of the common carp (*Cyprinus carpio* L.) innate immune system. Dev. Comp. Immunol. 30, 557-574.

Huttmann, A., Liu, S.L., Boyd, A.W., Li, C.L., 2001. Functional heterogeneity within rhodamine123(lo) Hoechst33342(lo/sp) primitive hemopoietic stem cells revealed by pyronin Y. Exp. Hematol. 29, 1109-1116.

Imagawa, T., Kitagawa, H. and Uehara, M., 1994 'Ultrastructure of blood vessels in the head kidney of the carp, *Cyprinus carpio*'. J. anat. 185, 521.

Katayama, Y., Battista, M., Kao, W.M., Hidalgo, A., Peired, A.J., Thomas, S.A., Frenette, P.S., 2006. Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. Cell 124, 407-421.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Yilmaz, O.H., Terhorst, C., Morrison, S.J., 2005. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 121, 1109-1121.

Kissa, K., Murayama, E., Zapata, A., Cortes, A., Perret, E., Machu, C., Herbomel, P., 2008. Live imaging of emerging hematopoietic stem cells and early thymus colonization. Blood 111, 1147-1156.

Kobayashi, I., Sekiya, M., Moritomo, T., Ototake, M., Nakanishi, T., 2006.

Demonstration of hematopoietic stem cells in ginbuna carp (*Carassius auratus langsdorfii*) kidney. Dev. Comp. Immunol. 30, 1034-1046.

Kobayashi, I., Moritomo, T., Ototake, M., Nakanishi, T., 2007. Isolation of side population cells from ginbuna carp (*Carassius auratus langsdorfii*) kidney hematopoietic tissues. Dev. Comp. Immunol. 31, 696-707.

Kobayashi, I., Kuniyoshi, S., Saito, K., Moritomo, T., Takahashi, T., Nakanishi, T., 2008a. Long-term hematopoietic reconstitution by transplantation of kidney hematopoietic stem cells in lethally irradiated clonal ginbuna crucian carp (*Carassius auratus langsdorfii*). Dev. Comp. Immunol. 32, 957-965.

Kobayashi, I., Saito, K., Moritomo, T., Araki, K., Takizawa, F., Nakanishi, T., 2008b. Characterization and localization of side population (SP) cells in zebrafish kidney hematopoietic tissue. Blood 111, 1131-1137.

Kobayashi, I., Kusakabe, H., Toda, H., Moritomo, T., Takahashi, T., Nakanishi, T., 2008c. *In vivo* characterization of primitive hematopoietic cells in clonal ginbuna crucian carp (*Carassius auratus langsdorfii*). Vet. Immunol. Immunopathol. 126, 74-82.

Kobayashi, I., Ono, H., Moritomo, T., Kano, K., Nakanishi, T., Suda, T., 2010. Comparative gene expression analysis of zebrafish and mammals identifies common regulators in hematopoietic stem cells. Blood 115, e1-9.

Kobayashi, I., Kobayashi-Sun, J., Kim, A.D., Pouget, C., Fujita, N., Suda, T., Traver, D., 2014. Jam1a-Jam2a interactions regulate haematopoietic stem cell fate through Notch signalling. Nature 512, 319-323.

Kollet, O., Dar, A., Shivtiel, S., Kalinkovich, A., Lapid, K., Sztainberg, Y., Tesio, M., Samstein, R.M., Goichberg, P., Spiegel, A., Elson, A., Lapidot, T., 2006. Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. Nat. Med. 12, 657-664.

Kunisaki, Y., Bruns, I., Scheiermann, C., Ahmed, J., Pinho, S., Zhang, D., Mizoguchi, T., Wei, Q., Lucas, D., Ito, K., Mar, J.C., Bergman, A., Frenette, P.S., 2013. Arteriolar

niches maintain haematopoietic stem cell quiescence. Nature 502, 637-643.

Langenau, D.M., Ferrando, A.A., Traver, D., Kutok, J.L., Hezel, J.P., Kanki, J.P., Zon, L.I., Look, A.T., Trede, N.S., 2004. *In vivo* tracking of T cell development, ablation, and engraftment in transgenic zebrafish. Proc. Natl. Acad. Sci. U. S. A. 101, 7369-7374.

Langenau, D.M., Zon, L.I., 2005. The zebrafish: a new model of T-cell and thymic development. Nat. Rev. Immunol. 5, 307-317.

Lapidot, T., Dar, A., Kollet, O., 2005. How do stem cells find their way home? Blood 106, 1901-1910.

Leemhuis, T., Yoder, M.C., Grigsby, S., Aguero, B., Eder, P., Srour, E.F., 1996. Isolation of primitive human bone marrow hematopoietic progenitor cells using Hoechst 33342 and Rhodamine 123. Exp. Hematol. 24, 1215–1224.

Li, L., Xie, T., 2005. Stem cell niche: structure and function. Annu. Rev. Cell Dev. Biol. 21, 605-631.

Lin, H.F., Traver, D., Zhu, H., Dooley, K., Paw, B.H., Zon, L.I., Handin, R.I., 2005. Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. Blood 106, 3803-3810.

Lorenz, E., Uphoff, D., Reid, T.R., Shelton, E., 1951. Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J. Natl. Cancer Inst. 12, 197-201.

Ma, D., Zhang, J., Lin, H.F., Italiano, J., Handin, R.I., 2011. The identification and characterization of zebrafish hematopoietic stem cells. Blood 118, 289-297.

Mahmud, N., Devine, S.M., Weller, K.P., Parmar, S., Sturgeon, C., Nelson, M.C., Hewett, T., Hoffman, R., 2001. The relative quiescence of hematopoietic stem cells in nonhuman primates. Blood 97, 3061–3068.

Matsuzaki, Y., Kinjo, K., Mulligan, R.C., Okano, H., 2004. Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. Immunity 20, 87-93.

Mendez-Ferrer, S., Michurina, T.V., Ferraro, F., Mazloom, A.R., Macarthur, B.D., Lira, S.A., Scadden, D.T., Ma'ayan, A., Enikolopov, G.N., Frenette, P.S., 2010. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466, 829-834.

Moore, K.A., Lemischka, I.R., 2006. Stem cells and their niches. Science 311, 1880-1885.

Morita, Y., Iseki, A., Okamura, S., Suzuki, S., Nakauchi, H., Ema, H., 2011. Functional characterization of hematopoietic stem cells in the spleen. Exp. Hematol. 39, 351-359 e353.

Moritomo, T., Asakura, N., Sekiya, M., Ototake, M., Inoue, Y., Nakanishi, T., 2004. Cell culture of clonal ginbuna crucian carp hematopoietic cells: differentiation of cultured cells into erythrocytes *in vivo*. Dev. Comp. Immunol. 28, 863-869.

Morrison, S.J., Spradling, A.C., 2008. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell 132, 598-611.

Morrison, S.J., Uchida, N., Weissman, I.L., 1995. The biology of hematopoietic stem cells. Annu. Rev. Cell Dev. Biol. 11, 35-71.

Murayama, E., Kissa, K., Zapata, A., Mordelet, E., Briolat, V., Lin, H.F., Handin, R.I., Herbomel, P., 2006. Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. Immunity 25, 963-975.

Nakamura-Ishizu, A., Takubo, K., Fujioka, M., Suda, T., 2014. Megakaryocytes are essential for HSC quiescence through the production of thrombopoietin. Biochem. Biophys. Res. Commun. 454, 353-357.

Nakanishi, T., Ototake, M., 1999. The graft-versus-host reaction (GVHR) in the ginbuna crucian carp, *Carassius auratus langsdorfii*. Dev. Comp. Immunol. 23, 15-26.

Nilsson, S.K., Johnston, H.M., Whitty, G.A., Williams, B., Webb, R.J., Denhardt, D.T., Bertoncello, I., Bendall, L.J., Simmons, P.J., Haylock, D.N., 2005. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive

hematopoietic progenitor cells. Blood 106, 1232-1239.

Orkin, S.H., Zon, L.I., 2008. Hematopoiesis: an evolving paradigm for stem cell biology. Cell 132, 631-644.

Orlic, D., Fischer, R., Nishikawa, S., Nienhuis, A.W., Bodine, D.M., 1993. Purification and characterization of heterogeneous pluripotent hematopoietic stem cell populations expressing high levels of c-kit receptor. Blood 82, 762–770.

Osawa, M., Hanada, K., Hamada, H., Nakauchi, H., 1996. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 273, 242-245.

Paw, B.H., Zon, L.I., 2000. Zebrafish: a genetic approach in studying hematopoiesis. Curr. Opin. Hematol. 7, 79-84.

Peled, A., Kollet, O., Ponomaryov, T., Petit, I., Franitza, S., Grabovsky, V., Slav, M.M., Nagler, A., Lider, O., Alon, R., Zipori, D., Lapidot, T., 2000. The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. Blood 95, 3289-3296.

Qian, H., Buza-Vidas, N., Hyland, C.D., Jensen, C.T., Antonchuk, J., Mansson, R., Thoren, L.A., Ekblom, M., Alexander, W.S., Jacobsen, S.E., 2007. Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. Cell Stem Cell 1, 671-684.

Quesada, J., Villena, M.I., Navarro, V. 2005. Ontogeny of the sea bass spleen (*Dicentrarchus labrax*): a light and electron microscopic study. J. Morphol. 221, 161–176.

Schofield, R., 1978. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells 4, 7-25.

Shechmeister, I.L., Watson, L.J., Cole, V.W., Jackson, L.L., 1962. The effect of x-irradiation on goldfish. I. The effect of x-irradiation on survival and susceptibility

of the goldfish, *Carassius auratus*, to infection by *Aeromonas salmonicida* and *Gyrodactylus* spp. Radiat. Res. 16, 89-97.

Soza-Ried, C., Hess, I., Netuschil, N., Schorpp, M., Boehm, T., 2010. Essential role of c-myb in definitive hematopoiesis is evolutionarily conserved. Proc. Natl. Acad. Sci. U. S. A. 107, 17304-17308.

Spangrude, G.J., Heimfeld, S.,Weissman, I.L., 1988. Purification and characterization of mouse hematopoietic stem cells. Science 241, 58–62.

Spangrude, G.J., Johnson, G.R., 1990. Resting and activated subsets of mouse multipotent hematopoietic stem cells. Proc. Natl. Acad. Sci. U. S. A. 87, 7433–7437.

Stier, S., Ko, Y., Forkert, R., Lutz, C., Neuhaus, T., Grunewald, E., Cheng, T., Dombkowski, D., Calvi, L.M., Rittling, S.R., Scadden, D.T., 2005. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. J. Exp. Med. 201, 1781-1791.

Sugiyama, T., Kohara, H., Noda, M., Nagasawa, T., 2006. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity 25, 977-988.

Swiers, G., Rode, C., Azzoni, E., de Bruijn, M.F., 2013. A short history of hemogenic endothelium. Blood Cells Mol. Dis. 51, 206-212.

Tamplin, O.J., Durand, E.M., Carr, L.A., Childs, S.J., Hagedorn, E.J., Li, P., Yzaguirre, A.D., Speck, N.A., Zon, L.I., 2015. Hematopoietic stem cell arrival triggers dynamic remodeling of the perivascular niche. Cell 160, 241-252.

Tang, Q., Abdelfattah, N.S., Blackburn, J.S., Moore, J.C., Martinez, S.A., Moore, F.E., Lobbardi, R., Tenente, I.M., Ignatius, M.S., Berman, J.N., Liwski, R.S., Houvras, Y., Langenau, D.M., 2014. Optimized cell transplantation using adult rag2 mutant zebrafish. Nat. Methods 11, 821-824.

Temmink, J. and Bayne, C., 1988. 'Ultrastructural characterization of leucocytes in the pronephros of carp (*Cyprinus carpio*, L.)'. Dev. Comp. Immunol. 11, 125-137.

Traver, D., Paw, B.H., Poss, K.D., Penberthy, W.T., Lin, S., Zon, L.I., 2003. Transplantation and *in vivo* imaging of multilineage engraftment in zebrafish bloodless mutants. Nat. Immunol. 4, 1238-1246.

Traver, D., Winzeler, A., Stern, H.M., Mayhall, E.A., Langenau, D.M., Kutok, J.L., Look, A.T., Zon, L.I., 2004. Effects of lethal irradiation in zebrafish and rescue by hematopoietic cell transplantation. Blood 104, 1298-1305.

Tsinkalovsky, O., Vik-Mo, A.O., Ferreira, S., Laerum, O.D., Fjose, A., 2007. Zebrafish kidney marrow contains ABCG2-dependent side population cells exhibiting hematopoietic stem cell properties. Differentiation 75, 175-183.

Uchida, N., Leung, F.Y., Eaves, C.J., 2002. Liver and marrow of adult mdr-1a/1b(-/-) mice show normal generation, function, and multi-tissue trafficking of primitive hematopoietic cells. Exp. Hematol. 30, 862–869.

Uchida, N., Dykstra, B., Lyons, K., Leung, F., Kristiansen, M., Eaves, C., 2004. ABC transporter activities of murine hematopoietic stem cells vary according to their developmental and activation status. Blood 103, 4487–4495.

Verfaillie, C., Blakolmer, K., McGlave, P., 1990. Purified primitive human hematopoietic progenitor cells with long-term *in vitro* repopulating capacity adhere selectively to irradiated bone marrow stroma. J. Exp. Med. 172, 502–509.

Winkler, I.G., Barbier, V., Nowlan, B., Jacobsen, R.N., Forristal, C.E., Patton, J.T., Magnani, J.L., Levesque, J.P., 2012. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. Nat. Med. 18, 1651-1657.

Winkler, I.G., Barbier, V., Wadley, R., Zannettino, A.C., Williams, S., Levesque, J.P., 2010. Positioning of bone marrow hematopoietic and stromal cells relative to blood flow *in vivo*: serially reconstituting hematopoietic stem cells reside in distinct nonperfused niches. Blood 116, 375-385.

Wognum, A.W., Eaves, A.C., Thomas, T.E., 2003. Identification and isolation of

hematopoietic stem cells. Arch. Med. Res. 34, 461-475.

Wolber, F.M., Leonard, E., Michael, S., Orschell-Traycoff, C.M., Yoder, M.C., Srour, E.F., 2002. Roles of spleen and liver in development of the murine hematopoietic system. Exp. Hematol. 30, 1010-1019.

Wolf, N.S., Kone, A., Priestley, G.V., Bartelmez, S.H., 1993. *In vivo* and *in vitro* characterization of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine 123 FACS selection. Exp. Hematol. 21, 614–622.

Yamazaki, S., Ema, H., Karlsson, G., Yamaguchi, T., Miyoshi, H., Shioda, S., Taketo, M.M., Karlsson, S., Iwama, A., Nakauchi, H., 2011. Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. Cell 147, 1146-1158.

Yoshihara, H., Arai, F., Hosokawa, K., Hagiwara, T., Takubo, K., Nakamura, Y., Gomei, Y., Iwasaki, H., Matsuoka, S., Miyamoto, K., Miyazaki, H., Takahashi, T., Suda, T., 2007. Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. Cell Stem Cell 1, 685-697.

Zapata, A., 1979. Ultrastructural study of the teleost fish kidney. Dev. Comp. Immunol. 3, 55-65.

Zapata, A., 1981. Lymphoid organs of teleost fist. II. Ultrastructure of renal lymphoid tissue of *Rutilus rutilus* and *Gobio gobio*. Dev. Comp. Immunol. 5, 685-690.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., Harris, S., Wiedemann, L.M., Mishina, Y., Li, L., 2003. Identification of the haematopoietic stem cell niche and control of the niche size. Nature 425, 836-841.

Zhao, M., Perry, J.M., Marshall, H., Venkatraman, A., Qian, P., He, X.C., Ahamed, J., Li, L., 2014. Megakaryocytes maintain homeostatic quiescence and promote post-injury regeneration of hematopoietic stem cells. Nat. Med. 20, 1321-1326.

Zhong, R.K., Astle, C.M., Harrison, D.E., 1996. Distinct developmental patterns of short-term and long-term functioning lymphoid and myeloid precursors defined by competitive limiting dilution analysis *in vivo*. J. Immunol. 157, 138-145.

Zhou, S., Schuetz, J.D., Bunting, K.D., Colapietro, A.M., Sampath, J., Morris, J.J., Lagutina, I., Grosveld, G.C., Osawa, M., Nakauchi, H., Sorrentino, B.P., 2001. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat. Med. 7, 1028–1034.

Zhou, S., Morris, J.J., Barnes, Y., Lan, L., Schuetz, J.D., Sorrentino, B.P., 2002. Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells *in vivo*. Proc. Natl. Acad. Sci. U. S. A. 99, 12339–12344.

Figure legends

Fig. 1. Preparation of triploid clones and tetraploid hybrids.

The clonal ginbuna carp is a triploid fish possessing three sets of chromosomes. The S3n strain has a unique character of reproduction. When eggs from S3n clones are inseminated with goldfish sperm, tetraploid hybrids are obtained. In contrast, when S3n eggs are inseminated with goldfish sperm inactivated by UV irradiation, triploid clonal fish are obtained.

Fig. 2. Ploidy analysis in S4n recipients

After transplantation of S3n kidney hematopoietic cells into S4n recipients, the percentage of donor-derived blood cells was determined by ploidy analysis. Erythrocytes (a) and leukocytes (b) were stained with propidium iodide and Hoechst 33342, respectively, and were examined by flow cytometry. Donor (triploid)- and recipient (tetraploid)-derived cells can be distinguished by their fluorescent intensity. Donor-derived leukocytes were sorted from the recipients and morphologically examined (c). Major types of leukocytes were detected in the recipients, including neutrophils (N), basophils (B), monocytes (M), thrombocytes (T), and lymphocytes (L). May-Gruenwald Giemsa. Bar=10 μm. Data adapted from Kobayashi et al., 2006.

Fig. 3. Isolation of SP cells from the ginbuna carp kidney.

Whole leukocytes were stained with Hoechst 33342 and analyzed by flow cytometry. Three different cell populations can be resolved, “S/G2/M”, “G0/G1” and “Side population (SP)”. The SP fraction was identified as a small streak of cells with low fluorescence. SP cells were morphologically uniform, composed of small lymphocyte-like cells. May-Gruenwald Giemsa. Data adapted from Kobayashi et al., 2007.

Fig. 4. The Hoechst staining profile of kidney hematopoietic cells in the ginbuna carp, zebrafish, and medaka.

Whole kidney leukocytes from the ginbuna carp, zebrafish, and medaka were stained with Hoechst 33342 and analyzed by flow cytometry. The fractions of the “SP” (lower gates) and “S/G2/M” (upper gates) are gated in each panel. Ginbuna carps showed the relatively higher percentage of the SP fraction, whereas medaka fish showed the higher percentage of the S/G2/M fraction.

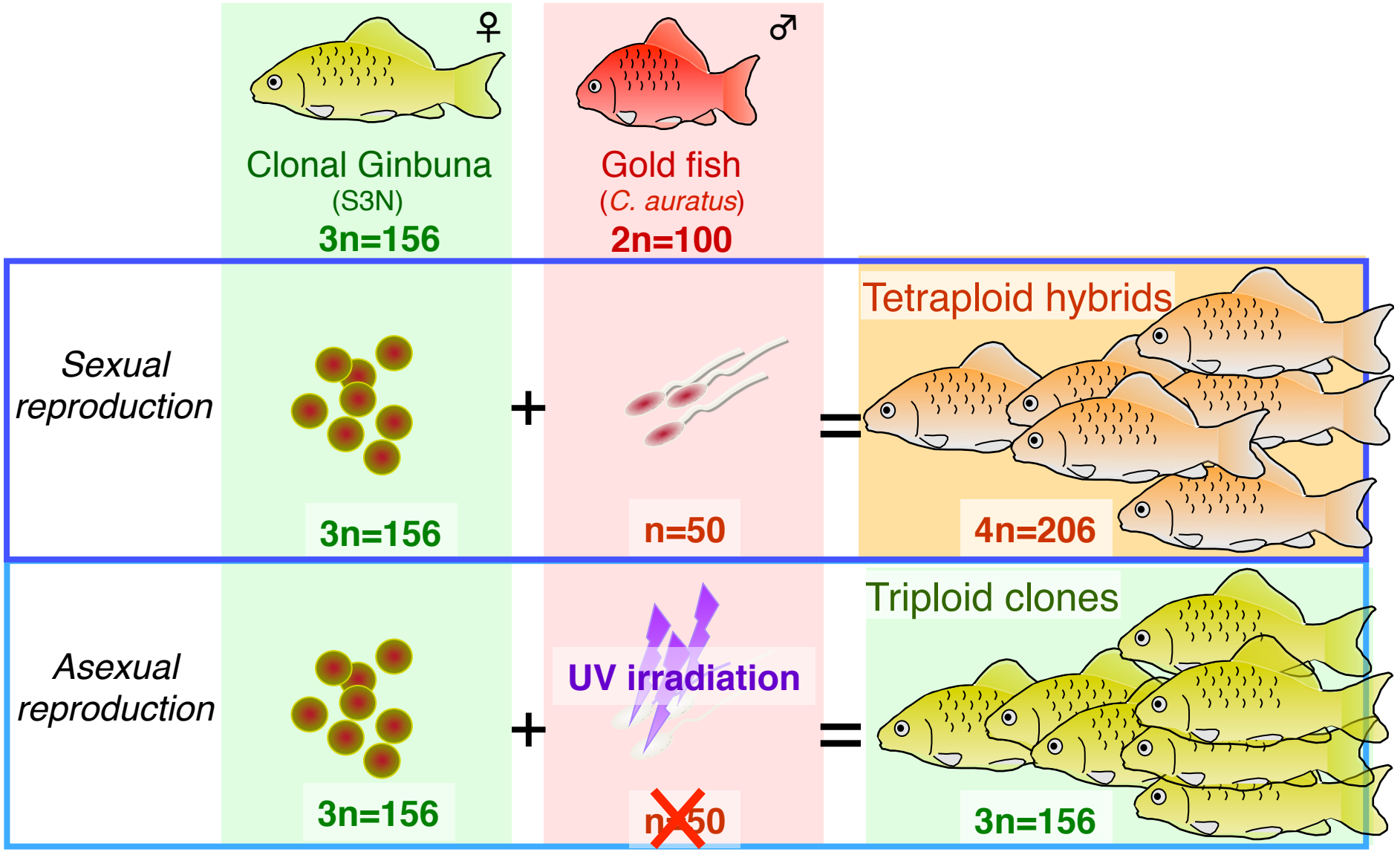
♀
♂
Clonal Ginbuna
(S3N)
3n=156
Gold fish
(C. auratus)
2n=100
Sexual reproduction
3n=156
+
n=50
=
Tetraploid hybrids
4n=206
Asexual reproduction
3n=156
+
UV irradiation
n=50
=
Triploid clones
3n=156

Figure 1

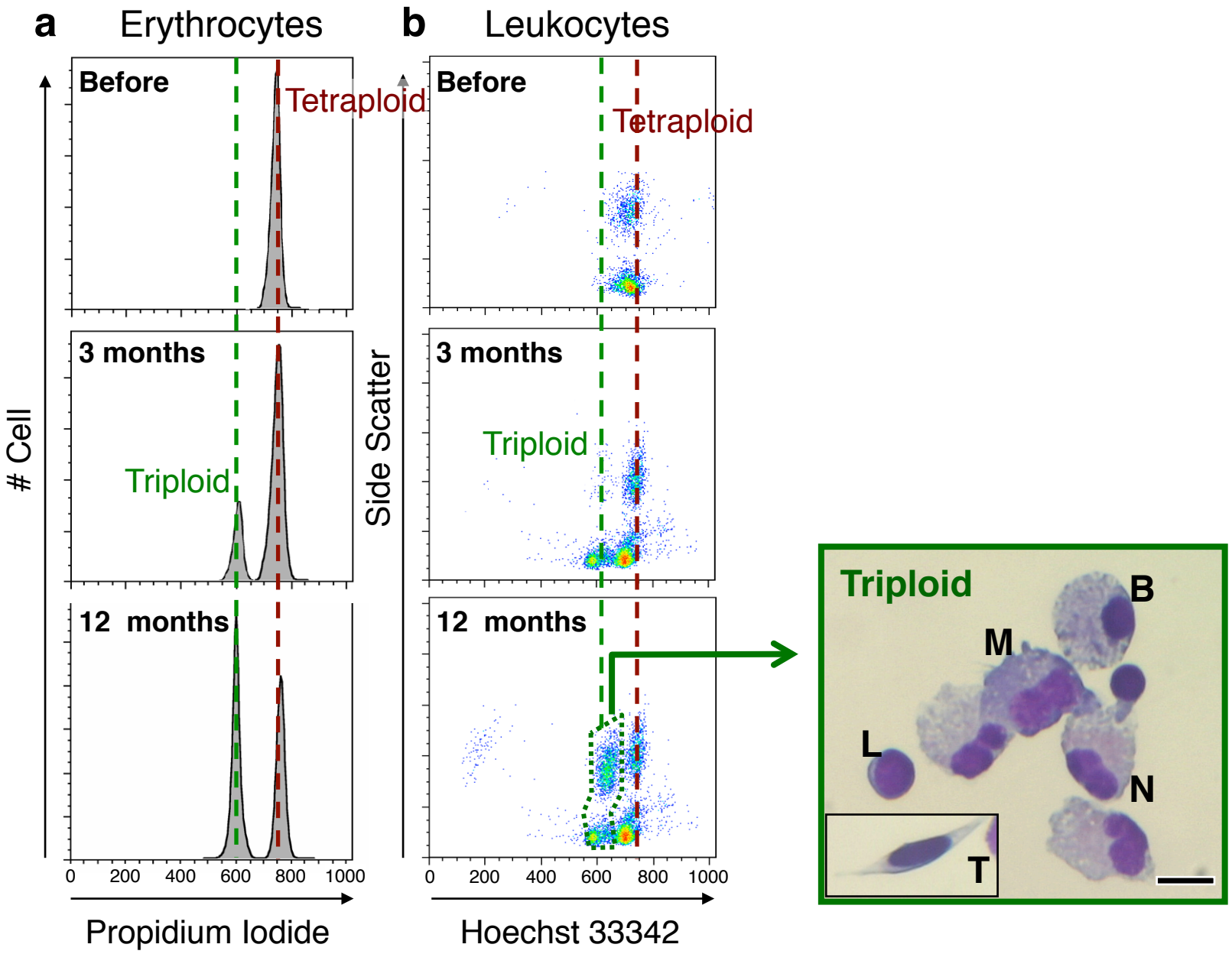
a
Erythrocytes
b
Leukocytes
Before
Tetraploid
3 months
Triploid
12 months
Cell
Side Scatter
0
200
400
600
800
1000
Propidium Iodide
Hoechst 33342
Triploid
B
M
L
N
T

Figure 2

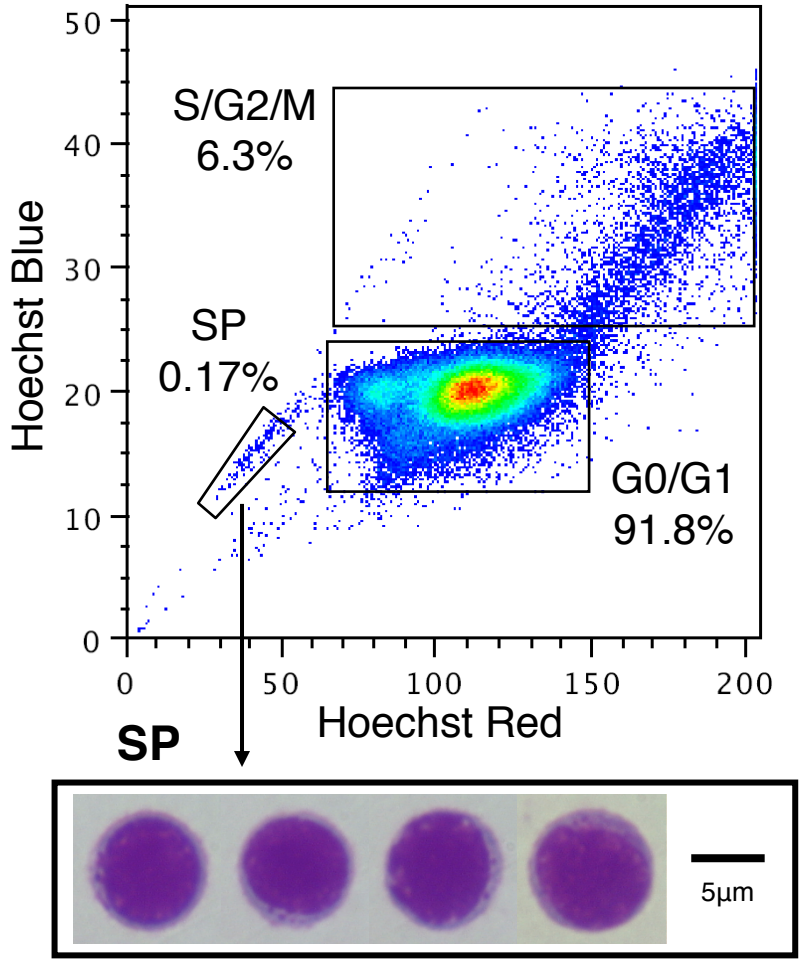
S/G2/M
6.3%
SP
0.17%
G0/G1
91.8%
Hoechst Blue
Hoechst Red
0
10
20
30
40
50
0
50
100
150
200
SP
5µm

Figure 3

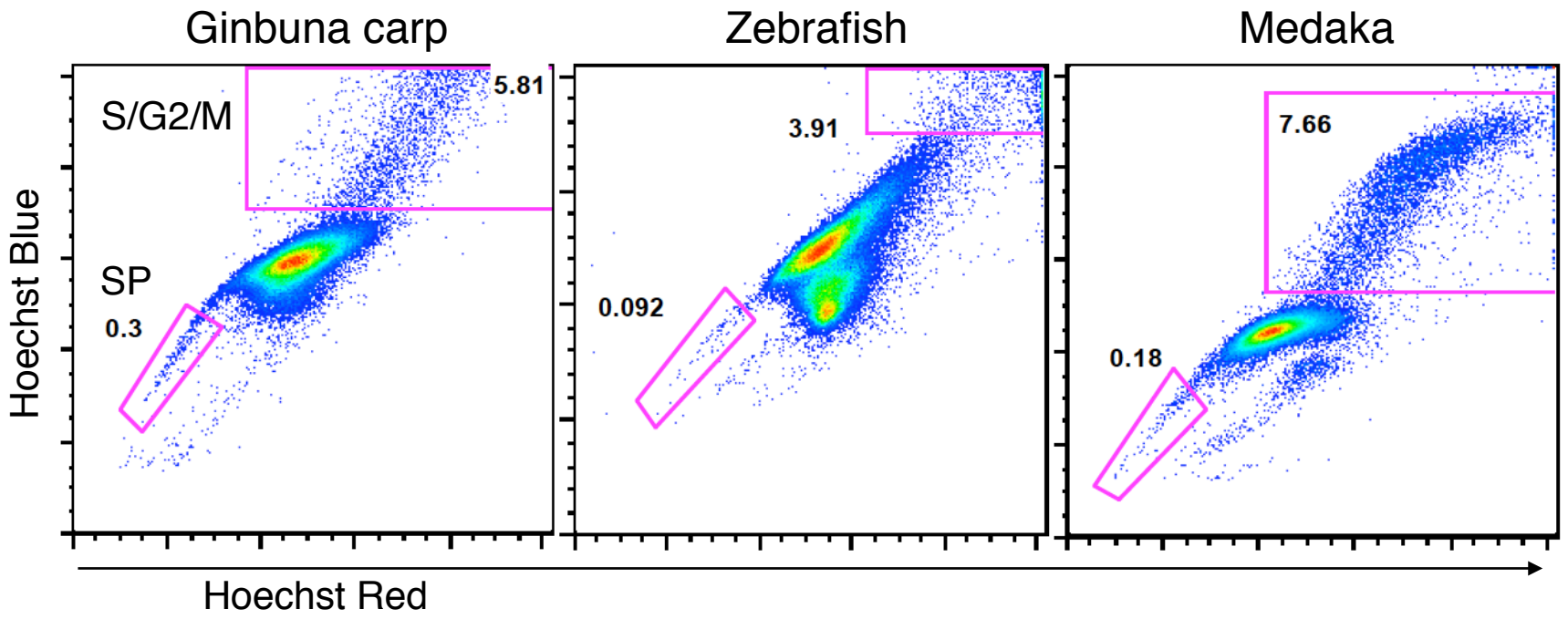
Ginbuna carp
Zebrafish
Medaka
S/G2/M
SP
5.81
0.3
3.91
0.092
7.66
0.18
Hoechst Blue
Hoechst Red

Figure 4