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Review

Isolation and characterization of hematopoietic stem cells in teleost fish

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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.

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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 and 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

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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 and Xie, 2005; Moore and Lemischka, 2006; Morrison and Spradling, 2008; Ema and 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.

1.1. Methods for assaying HSCs in teleost fish

1.1.1. 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 (*C. 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 and 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 β (*TCR-β*) 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 and 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 and 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