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Technical report

Co-culture of carp (*Cyprinus carpio*) kidney haematopoietic cells with feeder cells resulting in long-term proliferation of T-cell lineages

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ABSTRACT

To characterise fish haematopoietic stem/progenitor cells, it is necessary to develop a culture system that supports proliferation and differentiation of these cells. In the present study, we established cell lines from various tissues of carp (*Cyprinus carpio*) and ginbuna (*Carassius auratus langsdorfii*). By using these cell lines, we developed a culture system in which carp haematopoietic cells proliferated and were successively passaged. Cell lines from carp thymus (KoT), carp fin (KoF1) and ginbuna thymus (GTS6 and GTS9) were newly established. In addition to these cell lines, ginbuna fin (CFS) cell lines were also used as feeder layers. Kidney haematopoietic cells co-cultured with these feeder layers proliferated rapidly and were passaged over 20 times for more than 60 days. To characterise the proliferating cells, expression of marker genes for blood cell development were analysed. In the primary culture, marker genes for myeloid/erythroid progenitors (*gata1*), haematopoietic stem cells (*gata2*), neutrophils (*mpx*/*mpo*), B-cells (*IgH*) and T-cells (*lck*, *TCR*β and *gata3*) were detected by reverse transcriptase polymerase chain reaction (RT-PCR). Expression of most of the genes disappeared after the third passage, only T-cell marker genes were highly expressed after passages. These results indicate that multiple blood cells developed in the primary culture and then T-cell lineages dominantly proliferated after several passages.

Haematopoiesis is a complex process in which haematopoietic stem cells (HSCs) proliferate and differentiate into lineage-committed progenitors. These committed progenitor cells further differentiate to mature blood cells in a highly regulated manner.

Recently, several articles concerning teleost haematopoietic stem/progenitor cells have been published. Traver et al. and Langenau et al. demonstrated the presence of haematopoietic stem/progenitor cells in kidney haematopoietic tissues using the transplantation system of GFP-transgenic zebrafish (Langenau et al., 2004; Traver et al., 2003). We have also demonstrated the presence of kidney haematopoietic stem cells using the transplantation system of clonal ginbuna carp (*Carassius auratus langsdorfii*) and clonal ginbuna–gold fish hybrids (Moritomo et al., 2004). In our experiment, kidney haematopoietic cells were obtained from the ginbuna (triploid fish) and transplanted into the ginbuna–goldfish hybrids (tetraploid fish) (Kobayashi et al., 2006). We found donor-derived triploid blood cells including erythrocytes, thrombocytes, granulocytes and lymphocytes in the recipient for more than 18 months. These results indicate that long-term self-renewal and multipotent HSCs

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are present in the donor kidney (Kobayashi et al., 2006). More recently, we isolated HSCs from ginbuna and zebrafish kidney using side population (SP) analysis on the basis of Hoechst dye exclusion. The HSC activities of the SP cells were also demonstrated by their potential for long-term re-population and multi-lineage differentiation *in vivo* and gene expression analysis (Kobayashi et al., 2007; Kobayashi et al., 2008).

Characterisation of teleost haematopoietic stem/progenitor cells requires, in addition to the *in vivo* transplantation systems, developing an *in vitro* culture system that supports the proliferation and differentiation of haematopoietic stem/progenitor cells. In mammals, many stromal cell lines that are capable of supporting the development of blood cell lineages have been established. For instance, the mouse stromal cell lines of OP9 and Tst-4 have been shown to support the proliferation and differentiation of several blood lineages *in vitro* (Kawamoto et al., 1997; Kodama et al., 1994). By using these stromal cell lines, Kawamoto et al. established a multi-lineage progenitor (MLP) assay in which committed progenitor cells including commitment to T, B, erythroid and myeloid lineages were proliferated and differentiated in the presence of exogenous growth factors. Moreover, the MLP assay was capable of assaying the haematopoiesis from single stem/progenitor cell (Lu et al., 2002). In fish, however, such an *in vitro* system remains poorly developed.

In fish, several haematopoiesis-supporting cell lines have been developed. Diago et al. (1998) established a stromal cell line (TPS) from rainbow trout kidney. Ganassin et al. established a stromal cell line (RTS34st) from rainbow trout spleen (Ganassin and Bols, 1999). They reported that co-culture of rainbow trout haematopoietic cells with these cell lines resulted in long-term proliferation of haematopoietic cells. These results indicate that the stromal cell lines from the lympho-haematopoietic tissues could be used as efficient tools for investigating haematopoietic cell development even in fish.

In the present study, we tried to establish cell lines from various tissues of carp (*Cyprinus carpio*) and ginbuna. By using these cell lines as a feeder layer, we developed a culture system in which carp haematopoietic cells proliferated and successively transferred.

1. Material and methods

1.1. Fish and serum preparation

Carp (*C. carpio*) weighing 40–60 g (small carp) and 1–2 kg (large carp) were purchased from a commercial farm. Both fish were kept at 25 °C in a recirculation system with filtered water disinfected by ultraviolet light and fed with pelleted dry food once a day. Small carp were used for the cell harvesting as described below and for developing feeder cell lines. Large carp were used for serum preparation.

To prepare serum to be used as a culture media supplement, 30–40 ml of blood was obtained from the caudal vessel of large carp every 3 weeks. At each time, the sera were separated by centrifugation after allowing it to clot 2 h at room temperature and 2 h at 4 °C. The sera obtained from 2–3 carp were pooled and inactivated at 40 °C for 30 min. The pooled serum was then mixed with activated charcoal powder (Sigma) at a concentration of 10 mg ml^{-1} for 30 min with gentle rotation. The pooled serum was centrifuged and the supernatants were sterilised through a membrane filter (pore size 0.45 μm), and stored at −20 °C until use.

1.2. Establishment of cell lines from carp and ginbuna

Several cell lines were established from thymus and fin of carp. In short, thymus including surrounding tissues was dissected from a carp. A cell suspension was prepared by macerating the thymus on a stainless steel mesh with forceps in Eagle's minimum essential medium (MEM). These cells were then seeded into a flask, and cultured with enriched RDF (RPMI:DMEM:F12 in 2:1:1) medium (Kyokuto, Japan) containing 20% foetal calf serum and 2.5% carp serum (ERDF20/2.5) at 30 °C in 5% CO_2. The tail fin was also cut from a carp, disinfected in 70% ethanol for 20–30 s and rinsed twice in MEM. The fin was then cut into small fragments with scissors in a small amount of ERDF20/2.5, seeded into a flask and cultured with ERDF20/2.5 at 30 °C in 5% CO_2.

At suitable intervals (5–7 days), depending on the growth of cells, the thymus and fin cell cultures were refreshed by replacing half of medium with fresh medium. After the cells grew and formed a monolayer, the cells were trypsinised with 0.25% trypsin 1 mM ethylenediaminetetraacetic acid (EDTA) (Gibco) and reseeded into a new flask. After 10–12 serial passages, the culture media were changed from ERDF20/2.5 to ERDF20 (ERDF medium containing 20% foetal calf serum). At this step, the cells continuously grew without any morphological alterations, even in the absence of carp serum. After more than 20 serial passages (for more than 100 days), these cell lines were used for carp haematopoietic cell culture as feeder layer cells.

The cell lines from ginbuna (20–40 g, 1–2 years old) thymus were also established in the same manner for the carp thymus cell line, except that they were maintained in ERDF20 medium containing 5% ginbuna pooled serum.

1.3. Preparation of kidney haematopoietic cells from common carp

Carp haematopoietic cells were prepared as previously described (Moritomo et al., 1996). In short, carp kidney was macerated on a stainless steel mesh in 5 ml of MEM with forceps. Haematopoietic cells released from carp kidney were collected by a centrifugation. After discarding the supernatant, 2 ml of distilled water was added to the cell pellet to lyse mature erythrocytes and gently mixed several times with a pipette. Subsequently, 8 ml of MEM was added, and the cells were washed twice by centrifugation with MEM. Finally the cells were dispersed in ERDF20/2.5.

1.4. Co-culture of carp kidney haematopoietic cells with different cell lines

Cells of carp, ginbuna and mouse cell lines were seeded into new 25-cm^2 culture flasks and cultivated for 2–4 days