A simple method for isolation and culture of primary hepatocytes from *Salvelinus leucomaenis* (White-spotted Charr)

Katsuya Iuchi · Yasushi Arai · Kazuki Sasaki · Naoe Sato · Chikako Yokoyama · Toshiro Saruwatari · Hisashi Hisatomi

Received: 24 March 2020 / Accepted: 4 August 2020 / Published online: 10 August 2020
© Springer Nature B.V. 2020

Abstract White-spotted charr (*Salvelinus leucomaenis*, *S. l.*) is an anadromous cold water-adapted fish, distributed in the Far East. We have previously reported the complete mitochondrial DNA sequences of white-spotted charrs (*S. l. imbricus* and *S. l. pluvius*) in Japan. In general, fish hepatocytes are useful for cellular and biochemical studies of fish. In this study, we isolated hepatocytes from the liver of white-spotted charr and used basic methods, such as enzyme digestion and low centrifugation, to analyze the

molecular mechanisms involved in specific cellular responses. The isolated hepatocytes could be cultured at 5–20 °C but not 37 °C. The morphology of hepatocytes was altered in a temperature-dependent manner. The properties of hepatocyte were similar to those of living fish. Moreover, the proliferation rate and damage of isolated hepatocytes depended on the concentration of fetal bovine serum in the culture medium. Taken together, this study demonstrates that this simple method for isolation and culture of hepatocytes from white-spotted charr may be useful for other biochemical and cellular studies.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10616-020-00415-6>) contains supplementary material, which is available to authorized users.

K. Iuchi (✉) · K. Sasaki · H. Hisatomi
Department of Materials and Life Science, Faculty of
Science and Technology, Seikei University, 3-3-1
Kichijojikitamachi, Musashino-shi, Tokyo 180-8633,
Japan
e-mail: iuchi@st.seikei.ac.jp;
katsuyaiuchi0707@gmail.com

Y. Arai · N. Sato
Seikei Junior and Senior High School, 3-10-13
Kichijojikitamachi, Musashino-shi, Tokyo 180-8633,
Japan

C. Yokoyama
Department of Biochemical Engineering, Graduate
School of Science and Engineering, Yamagata University,
4-3-16 Jonan, Yonezawa, Yamagata 992-8510, Japan

T. Saruwatari
Atmosphere and Ocean Research Institute, The University
of Tokyo, 5-1-5 Kashiwanoha, Kashiwa,
Chiba 277-8564, Japan

Keywords Experimental model · Cell culture · Hepatocytes · White-spotted charr

Introduction

Salvelinus leucomaenis (*S. l.*), or white-spotted charr, is an anadromous freshwater fish. The white-spotted charr is distributed from Honshu to Kamchatka Peninsula (Hosoya 2013). The white-spotted charr has been classified into four subspecies (*S. l. imbricus*, *S. l. japonicus*, *S. l. leucomaenis*, and *S. l. pluvicus*) based on zoogeographic patterns and morphological characteristics (Nakabo 2000). We recently reported the complete mitochondrial DNA sequences of two white-spotted charrs (*S. l. imbricus*, *S. l. pluvius*) in Japan, as well as those of other charrs (Arai et al. 2019). Recently, the habitat of *S. leucomaenis* in Japan has been threatened by global warming, which induces physiological stress in cold-water fish. Thus, *S. leucomaenis* is potentially vulnerable to the ongoing climate change and global warming. Takegawa et al. reported that catchment area, slope inclination, distance to the river mouth, and estimated ground water temperature were key factors for potential habitats (Takegawa et al. 2017).

In the field of ichthyology, liver has been used widely to investigate the fish health conditions. Especially, liver weight was used as a biological marker for reproduction of rainbow trout, *Oncorhynchus mykiss* (Nomura 1963). Hepatosomatic indices (HIS) have been used to estimate the fish reproduction (Ishii 1971). The role of estrogen has been investigated in the liver of *Gasterosteus aculeatus aculeatus* L., *Misgurnus anguillicaudatus*, *Oryzias latipes* (Egami 1955; Kobayashi 1953; Oguro 1956).

Methods to isolate intact and functioning cells from the liver of mammals became available during the late 1960s (Braunbeck and Segner 2000). Primary cell cultures are useful for studying the biochemical and cellular mechanisms underlying human diseases. Human primary liver cancer-derived organoid cultures are used for liver cancer modeling and anti-cancer drug screening (Broutier et al. 2017). Primary human hepatocytes are widely used for basic biological research and pharmaceutical studies (Hewitt et al. 2007).

The fish cell lines are versatile tools in toxicology and ecotoxicology applications (Bols et al. 2005). Birnbaum et al. were the first to report the use of goldfish hepatocytes in research studies (Birnbaum et al. 1976). To date, a large number of studies using fish hepatocytes have been reported (Segner 1998). Cell cultures of fish-derived epithelial cells and hepatocytes are useful for ichthyological research (Busby et al. 2002; Hayashi 2002; Lee et al. 1993; Ostrander et al. 1995; Pesonen et al. 2000). Fish primary hepatocytes have been used to study the metabolism of xenobiotics, the formation of toxic products, chemically-induced DNA damage, the induction of enzymes, peroxisome proliferation, the effects on hormone-signaling systems, etc. (Pesonen et al. 2000). The isolation and cultivation of teleost hepatocytes are reviewed by Segner, Helmut (Segner 1998). The isolation and culture conditions, e.g., cultivation temperature, media, and sera, are species-dependent (Braunbeck and Segner 2000). The requirements for a primary culture system for hepatocytes include an optimized isolation protocol and the provision of culture conditions (medium, cellular microenvironment) that are optimal for cell survival and functioning (Braunbeck and Segner 2000).

In the present study, we isolated primary liver cells using a simple method with enzyme digestion and low centrifugation and demonstrated that the morphology and cell growth of hepatocytes are dependent on the culture conditions (e.g. culture temperature, serum concentration, and dish surface).

Materials and methods

Reagents

All chemicals (unless otherwise stated) were obtained from Fujifilm Wako Pure Chemical Industries (Tokyo, Japan) or Sigma (St. Louis, MO, USA).

Ethical statement

The care and use of experimental animals complied with the animal welfare laws, guidelines and policies as approved by the Institutional Laboratory Animal Care and Use Committee of Seikei University.

Fig. 1 Schematic representation of the simple method used for isolating primary hepatocytes. Hepatocytes were isolated from the liver of white-spotted charr using a simple method, as described in the materials and methods section

Fish

White-spotted charr, *Salvelinus leucomaenis*, were purchased from a fish farm in Tokyo. The fish were kept in glass aquaria containing aerated tap water at a temperature of 14.5 °C.

Primary cell culture

Hepatocytes were cultured in Leibovitz's L-15 (L-15) medium (128-06075; Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (F7524;

Sigma-Aldrich, MO, USA) and 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (161-23181; Wako, Osaka, Japan) at temperatures in the range of 5–37 °C under atmospheric pressure.

Hepatocyte preparation from the liver of white-spotted charr

White-spotted charr were incubated on ice for about 1 h until a semi-hibernation state was reached. The fish cavity was opened using a scalpel and scissors. The liver was removed from the cavity, washed with cold PBS several times, and perfused with Accutase (Innovative Cell Technologies, Inc.) three times or more to remove blood from the vessels. The perfusion medium comprised Accutase solution in Dulbecco's PBS containing 0.5 mM EDTA and phenol red. The liver was subsequently cut with scissors to 1 mm³ pieces. The liver pieces were treated with Accutase for 60 min at 20 °C on a gyratory shaker and filtered through a nylon mesh (59 µm pore size) to remove large cell aggregates. The filtrate was centrifuged for 1 min at 50 × g and the cell pellets were resuspended in L-15 medium containing antibiotics and FBS.

Gelatin-coating of culture dishes

Gelatin-coating of culture dishes was performed as previously described (Iuchi et al. 2020). Briefly, 0.2% of gelatin solution was added into each dish and the gelatin solution-covered dishes were incubated at 37 °C to polymerize for 30 min. The excessive gelatin solution was aspirated, and the dishes were incubated again until it dried.

Observation of primary hepatocytes from *Salvelinus leucomaenis*

The hepatocytes were plated on the various polystyrene plates, cultured for the indicated times, and observed using a microscope (CKX53-21PH; Olympus systems, Germany or Leica DM IL LED; Leica microsystems, Wetzlar, Germany).

Fig. 2 Blood and primary liver cells at each step of the process. **a** The figures depict blood and primary liver cells in the steps described in Fig. 1. **b** Cell death of primary hepatocyte. The

cells were stained with Hoechst33342 and propidium iodide as described in the Materials and Methods section

Cell viability of primary hepatocytes from *Salvelinus leucomaenis*

Cell viability was measured using a cell counting kit (Kit-8, CCK-8, Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's protocol. Briefly, the cells were cultured in various conditions and the CCK-8 reagent was added. The cells were then incubated for 3–6 h at 20 °C. Absorbance was measured at 450 nm using a microplate reader (Food Mark, Bio-Lad, CA, USA).

Cell death of primary hepatocytes

The cell death assay was performed by Hoechst 33342 (HO) and propidium iodide (PI) staining. Cells were stained with 2 µg/mL HO and 2 µg/mL PI and observed under a fluorescence microscope (Leica DM IL LED, Leica Microsystems, Wetzlar, Germany). Cell death was measured by counting the PI-positive cells.

Statistical analysis

The data shown in the figures represent mean values $\pm$ standard deviation (SD) of 4–8 independent

experiments. The data were statistically evaluated by one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference test. The statistical significance at different *P*-values is indicated in each figure.

Results

Isolation of primary cells from *Salvelinus leucomaenis* liver

To isolate the primary cultured cells from *Salvelinus leucomaenis* liver, we used the method shown in Fig. 1. First, each fish was incubated for 1 h on ice until it stopped moving. After dissection, the liver was perfused with Accutase and its color subsequently changed from dark-red to light-brown. The perfusion was performed with using a 1 mL sterile syringe and needle (27 g $\times$ 1/2). As shown in Fig. 2a, the Accutase solution oozed out of the liver, removing the blood from the organ (Fig. 2a). After enzyme digestion and filtration, the blood remained in the cell suspensions. To remove the blood, the cell suspension was centrifuged at low-speed (50 $\times$ g) for 1 min several times. After centrifugation, the cell suspension mainly

Fig. 3 The morphology of hepatocytes during long-term culture. **a** Isolated hepatocytes were cultured on dishes for at the indicated times at 10 °C and observed by microscopy. **b** Cell

death of primary hepatocyte. The cells were stained with Hoechst33342 and propidium iodide as described in the Materials and Methods section

contained liver cells (Fig. 2a). As shown in Fig. 2b, most cells (> 90%) were alive on the culture dish (Fig. 2b).

Culture of primary cells of *Salvelinus leucomaenis* liver

As shown in Fig. 3, primary liver cells survived at 10 °C for > 2 weeks (Fig. 3a, b). At week 1, the hepatocytes formed 3-dimensional multicellular spheroids. The cells assumed a 2- or 3-dimensional shape at about 2 weeks in the culture dish. In addition, hepatic stellate cells could sometimes be observed in the isolated primary cells (Fig. S1). This suggested that the primary liver cells isolated by this simple method were mostly hepatocytes, with the exception of a few non-hepatocytes.

Temperature-dependent properties of the primary liver cell culture

White-spotted charr inhabits in rivers where the water temperature is less than 15 °C. We investigated the effect of culture temperature on hepatocyte cell growth. A range of culture temperatures were examined to choose the appropriate temperature for optimal fish growth. As shown in Fig. 4, the hepatocytes survived at temperatures ranging from 5 to 20 °C, while decreased cell proliferation was observed at 37 °C (Fig. 4a). Moreover, the hepatocytes could survive in 3-dimensional or 2-dimensional shapes on the culture dish at 5 or 20 °C (Fig. 4b). Interestingly, the morphology of the hepatocytes changed at 37 °C when the cells shrunk. The hepatocytes were vulnerable to temperature elevation. These results show that

Fig. 4 Effect of culture temperature on cell growth and morphology. **a** Hepatocytes were cultured at the indicated temperatures for 4 days and cell growth was measured by the CCK-8 assay, as described in the materials and methods section. (N = 8). ** $P < 0.01$, *** $P < 0.001$ **b** Hepatocytes were cultured at the indicated temperatures for 3 days and cell morphology was observed by microscopy

the physiological properties of primary cells are similar to those of living fish.

FBS-dependent cell growth of primary hepatocytes

Growth factors, such as the epidermal growth factor (EGF), hepatocyte growth factor (HGF), and fibroblast growth factors 1 and 2 (FGF1 and FGF2), directly modify the growth rate of mammalian hepatocytes (Bowen et al. 2014). We investigated the effect of serum on the growth of hepatocytes. As shown in Fig. 5, serum enhanced cell growth in a concentration-dependent manner (Fig. 5a, b). Thus, growth factors are as necessary for the growth of fish hepatocytes as they are for the growth of primary mammalian cells. Moreover, as shown in Fig. 5c, FBS protected hepatocyte from cell damage (Fig. 5c).

Effect of gelatin-coating on cell morphology and cell growth

Cultured cells attach more easily to a polystyrene culture dish coated with gelatin than to a non-coated dish. We have recently published the different morphologies of human embryonic kidney (HEK293T) cells in various culture dish conditions (Iuchi et al. 2020). Particularly, we examined whether gelatin affected the morphology and cell growth of hepatocytes. As shown in Fig. 6, culturing the cells on gelatin-coated dishes slightly inhibited the transition from a 3-dimensional to a 2-dimensional shape (Fig. 6).

Discussion

In this study, we isolated white-spotted charr hepatocytes using Accutase digestion and low-speed centrifugation. The primary hepatocytes formed 3-dimensional multicellular spheroids and 2-dimensional hexagonal conformations. Moreover, the proliferation of primary hepatocytes depended on both the concentration of fetal bovine serum in the culture medium and the culture temperature. This simple method of hepatocyte isolation may also be useful for other biochemical and cell culture studies.

Culture medium formulation and vessel material have significant effects on the induction of cytochrome P450 (CYP) enzymes in cultured rat hepatocytes (LeCluyse et al. 1999). High levels of organochlorine contaminants induce DNA double-strand breaks in arctic charr (*Salvelinus alpinus*) from Lake Ellasjøen, Bjørnøya (Norwegian Arctic) (Neerland et al. 2019). Hepatocyte isolated with our method may be used for analysis of DNA damage by chemicals in more detail.

Yanhong et al. reported the best culture conditions for *Cyprinus carpio* hepatocytes (Yanhong et al. 2008). This report showed that the best medium was M199 (cultured in 5% CO₂) or L-15 (cultured without 5% CO₂), and the optimal culture temperature was 27 °C. The optimal culture conditions for hepatocytes are fish type-dependent.

Hayashi reported that insulin is essential for the attachment and spreading of eel hepatocytes (Hayashi and Kumagai 2008). Eel hepatocytes cultured in an EGF or growth hormone-deficient medium had a

Fig. 5 Effect of fetal bovine serum on hepatocyte cell growth. **a** Hepatocytes were cultured in the presence of fetal bovine serum (FBS) at the indicated percentage for 7 days at 10 °C. Cell growth was measured by the CCK-8 assay, as described in the materials and methods section. (N = 4). ** $P < 0.01$, ***

$P < 0.001$ **b** Hepatocytes were cultured in the presence of fetal bovine serum at the indicated percentage for 7 days at 10 °C. The cells were observed by microscopy. **c** Hepatocyte was cultured in the absence or presence of FBS for 6 h at 10 °C and the cell morphology were observed by microscopy

normal morphology, but the EGF-deficient medium caused an approximately 60% decrease in the cell number compared to the control. In the present study, FBS enhanced cell growth (Fig. 5), suggesting that growth factors, such as EGF and HGF, are essential for cell growth.

The appetite of white-spotted charr decreased at temperatures > 24 °C, while a high water temperature (> 26 °C) resulted in the fish dying (Takami et al.

1997). In the present study, we elucidated the proper culture conditions for white-spotted charr-derived hepatocytes. The isolated hepatocytes could grow in culture at 5–20 °C but not 37 °C. The morphology of the hepatocytes cultured at 5 °C was different from that observed in other conditions. Further analyses of white-spotted charr hepatocytes may clarify the molecular mechanism underlying fish.

Fig. 6 Effect of different dish conditions on hepatocyte cell growth. Hepatocytes were cultured on non-coated or gelatin-coated dishes for 7 or 14 days at 10 °C. The cells were observed by microscopy

In conclusion, this study demonstrates that this simple method for isolation and culture of hepatocytes from white-spotted charr may be useful for conducting other biochemical and cellular studies. For example, these primary liver cells may be useful for analyzing the effect of global warming in white-spotted charr at the molecular level.

Acknowledgements We thank Mr. Yuji Tasaki, Ms. Rika Imaizumi and Mr. Tetsushi Seki for experimental support. We thank Prof. Naomi Kamimura for scientific advice.

Authors' contributions KI, YA, NS, CY, TS and HH conceived and designed the experiments. KI, YA, and KS performed the experiments and contributed the data analysis. KI, YA and TS wrote the manuscript. All authors contributed critically to drafts and gave final approval for submitting.

Data availability statement The data that support the findings of this study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

Competing financial interests The all authors declare no conflicts of interest.

References

- Arai Y, Yokoyama C, Nagase K, Suwa M, Ogawa Y, Iuchi K, Hisatomi H (2019) Complete mitochondrial DNA sequences of two endemic subspecies, *Salvelinus leucomaenis imbricus* and *Salvelinus leucomaenis pluvius* (Salmonid, White spotted charr) in Japan. Mitochondrial DNA Part B 4:1524–1525. <https://doi.org/10.1080/23802359.2019.1601517>
- Birnbaum MJ, Schultz J, Fain JN (1976) Hormone-stimulated glycogenolysis in isolated goldfish hepatocytes. Am J Physiol 231:191–197. <https://doi.org/10.1152/ajplegacy.1976.231.1.191>
- Bols N, Dayeh V, Lee L, Schirmer K (2005) Use of fish cell lines in the toxicology and ecotoxicology of fish. Piscine cell lines in environmental toxicology, vol 6. Biochemistry and molecular biology of fishes. Elsevier, New York, pp 43–84. [https://doi.org/10.1016/s1873-0140\(05\)80005-0](https://doi.org/10.1016/s1873-0140(05)80005-0)
- Bowen WC, Michalopoulos AW, Orr A, Ding MQ, Stolz DB, Michalopoulos GK (2014) Development of a chemically defined medium and discovery of new mitogenic growth factors for mouse hepatocytes: mitogenic effects of FGF1/2 and PDGF. PLoS ONE 9:e95487. <https://doi.org/10.1371/journal.pone.0095487>
- Braunbeck T, Segner H (2000) Isolation and cultivation of teleost hepatocytes. Springer, Berlin, pp 49–71. https://doi.org/10.1007/978-94-017-3345-8_6
- Broutier L et al (2017) Human primary liver cancer-derived organoid cultures for disease modeling and drug screening. Nat Med 23:1424–1435. <https://doi.org/10.1038/nm.4438>
- Busby ER, Cooper GA, Mommsen TP (2002) Novel role for prostaglandin E2 in fish hepatocytes: regulation of glucose metabolism. J Endocrinol 174:137–146. <https://doi.org/10.1677/joe.0.1740137>

- Egami N (1955) Effect of estrogen and androgen on the weight and structure of the liver of the fish, *Oryzias latipes*. *Annot Zool Jpn* 28:79–85
- Hayashi S (2002) Regulation of synthesis and secretion of the lipoprotein by cultured eel hepatocytes. *Fish Sci* 68:1213–1216. https://doi.org/10.2331/fishsci.68.sup2_1213
- Hayashi S, Kumagai A (2008) Studies on eel liver functions using perfused liver and primary cultured hepatocytes. *Aqua-BioSci Monogr*. <https://doi.org/10.5047/absm.2008.00102.0001>
- Hewitt NJ et al (2007) Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev* 39:159–234. <https://doi.org/10.1080/03602530601093489>
- Hosoya K (2013) *Salmonidae*. Fishes of Japan with pictorial keys to the species, 3rd edn. Tokai University Press, Tokai
- Ishii K (1971) Morphological changes in the liver of the Kokanee, *Oncorhynchus nerka*, accompanied with sexual maturation. *Bull Fac Fish Hokkaido Univ* 22:215–220
- Iuchi K, Oya K, Hosoya K, Sasaki K, Sakurada Y, Nakano T, Hisatomi H (2020) Different morphologies of human embryonic kidney 293T cells in various types of culture dishes. *Cytotechnology* 72:131–140. <https://doi.org/10.1007/s10616-019-00363-w>
- Kobayashi H (1953) Effect of estrone upon the structure, weight and fat content of the liver in the fish, *Misgurnus anguillicaudatus*. *Annot Zool Jpn* 26:213–216
- LeCluyse E, Bullock P, Madan A, Carroll K, Parkinson A (1999) Influence of extracellular matrix overlay and medium formulation on the induction of cytochrome P-450 2B enzymes in primary cultures of rat hepatocytes. *Drug Metab Dispos* 27:909–915
- Lee LE et al (1993) Development and characterization of a rainbow trout liver cell line expressing cytochrome P450-dependent monooxygenase activity. *Cell Biol Toxicol* 9:279–294. <https://doi.org/10.1007/bf00755606>
- Nakabo T (2000) *Fishes of Japan with pictorial keys to the species*, Japanese edition I. Tokai Univ. Press, Tokyo
- Neerland ED, Bytingsvik J, Nikiforov VA, Evenset A, Krokje A (2019) DNA Double-Strand Breaks in Arctic Char (*Salvelinus alpinus*) from Bjornoya in the Norwegian Arctic. *Environ Toxicol Chem* 38:2405–2413. <https://doi.org/10.1002/etc.4546>
- Nomura M (1963) Studies on reproduction of rainbow trout, *Salmo gairdneri*, with special reference to egg taking. V. Development of gonads and size of fish spawned firstly. *Nippon Suisan Gakkaishi* 29:976–984
- Oguro C (1956) Some observations on the effect of estrogen upon the liver of the three-spined stickleback, *Gasterosteus aculeatus aculeatus* L. *Environ Health Perspect* 29:19–22
- Ostrander GK et al (1995) Long-term primary culture of epithelial cells from rainbow trout *Oncorhynchus mykiss* liver. *In Vitro Cell Dev Biol Anim* 31:367–378. <https://doi.org/10.1007/bf02634286>
- Pesonen M, Teivainen P, Lundstrom J, Jakobsson E, Norrgren L (2000) Biochemical responses of fish sac fry and a primary cell culture of fish hepatocytes exposed to polychlorinated naphthalenes. *Arch Environ Contam Toxicol* 38:52–58. <https://doi.org/10.1007/s002449910007>
- Segner H (1998) Isolation and primary culture of teleost hepatocytes. *Compar Biochem Physiol Part A* 120:71–81. [https://doi.org/10.1016/s1095-6433\(98\)10012-0](https://doi.org/10.1016/s1095-6433(98)10012-0)
- Takami T, Kitano F, Nakano S (1997) High Water Temperature Influences on Foraging Responses and Thermal Deaths of Dolly Varden < i>Salvelinus malma </i> and White-spotted Charr < i>S. leucomaenis </i> in a Laboratory Fisheries science 63:6–8 <https://doi.org/10.2331/fishsci.63.6>
- Takegawa Y, Kawaguchi Y, Mitsuhashi H, Taniguchi Y (2017) Implementing species distribution models to predict the impact of global warming on current and future potential habitats of white-spotted char *Salvelinus leucomaenis* for effective conservation planning in Japan. *Jpn J Conserv Ecol* 22:121–134
- Yanhong F, Chenghua H, Guofang L, Haibin Z (2008) Optimization of the isolation and cultivation of *Cyprinus carpio* primary hepatocytes. *Cytotechnology* 58:85–92. <https://doi.org/10.1007/s10616-008-9169-5>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.