Title	Production of loach (<i>Misgurnus anguillicaudatus</i>) germ-line chimera using transplantation of primordial germ cells isolated from cryopreserved blastomeres
Author(s)	Yasui, G.S.; Fujimoto, T.; Sakao, S.; Yamaha, E.; Arai, K.
Citation	Journal of animal science, 89(8), 2380-2388 https://doi.org/10.2527/jas.2010-3633
Issue Date	2011-08-01
Doc URL	http://hdl.handle.net/2115/49756
Type	article (author version)
File Information	Blastomere-cryopreservationand-chimeras-in-fish-1-final-revised.pdf

[Instructions for use](#)

1 **Running title:** Cryopreservation and chimerism in fish

2
3 **Title:** Production of loach (*Misgurnus anguillicaudatus*) germ-line chimera using transplantation
4 of primordial germ cells isolated from cryopreserved blastomeres¹

5
6 G.S. Yasui^{*1}, T. Fujimoto^{*}, S. Sakao[†], E. Yamaha[‡], and K. Arai^{*}

7
8 ^{*} Laboratory of Aquaculture Genetics and Genomics, Division of Marine Life Sciences, Graduate
9 School of Fisheries Sciences, Hokkaido University, 3-1-1- Minato-cho, Hakodate, Hokkaido,
10 041-8611, Japan.

11
12 [†] Department of Molecular Cell Biology, Research Center for Elderly Nutrition and Development,
13 Mukogawa Women's University, 6-46, Ikebiraki-cho, Nishinomiya, Hyogo 663-8558, Japan

14
15 [‡] Nanae Fresh Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido
16 University, 2-9-1, Sakura, Nanae, Kameda, Hokkaido, 041-1105, Japan.

17

¹ This study was supported in part by Grants-in-Aid for the 21st Century COE (Center of Excellence) Program (K-2, 2004-2008 fiscal year) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), and Scientific Research (B) (no.18380108 and 21380114) from the Japan Society for the Promotion of Science (JSPS). This study was also supported by PROBRAIN (Promotion of Basic Research Activities for Innovative Biosciences) from the Bio-oriented Technology Research Advancement Institution.

² Corresponding author: george@fish.hokudai.ac.jp, georgeyasui@yahoo.com

ABSTRACT

An efficient procedure for cryopreservation of fish blastomeres followed by restoration through germ-line chimera formation was established. Blastomeres of the loach (*Misgurnus anguillicaudatus*) were cryopreserved in 250- μ L straws in Eagle's minimum essential medium (MEM) with various concentrations of dimethyl-sulfoxide (DMSO; 0, 5, 10, 15, and 20%), and the best concentration was combined with glycerol (1, 2, and 4%) and external cryoprotectants (1 or 2% sucrose; 2, 5, or 10% fetal bovine serum; 1 or 2% bovine serum albumin). Post-thaw viability of the blastomeres was used to optimize cryopreservation conditions. Donor blastomeres were injected with zebrafish GFP-*nos1* 3'UTR mRNA and biotin dextran prior to cryopreservation in the optimal freeze medium. Host embryos were injected with zebrafish DsRed-*nos1* 3'UTR mRNA and reared to the blastula stage. Donor blastomeres were thawed at 25°C for 10 s and transplanted to the host embryos either immediately or after incubation for 16 h at 20°C. Donor and host primordial germ cell migration was visualized with fluorescent imaging during the early stages of embryogenesis, and also by histology in 4-d-old embryos. Transplantation of blastomeres immediately after thawing gave lower hatching rates (~3%) and generated a low percentage of germ-line chimeras (~1.1%). In contrast, incubation of cryopreserved sample for 16 h followed by transplantation of the GFP-positive blastomeres improved the hatching rate to 90%, and successfully produced presumable germ-line chimeras at a rate of 16.5%. The improved survival rates and germ-line chimerism may be an effective method for gene banking and subsequent reconstitution of endangered fish genotypes.

Keywords: aquaculture, cryobank, embryo, gamete, germ cell, germplasm, loach, teleost.

INTRODUCTION

Genebanking is of importance for both farmed and wild species as a means to preserve endangered populations and reconstitute unique genotypes. In fish, a large number of species or strains are endangered (Hiemstra et al., 2006), and farmed stocks are derived from few genotypes from wild populations. Thus, germplasm preservation may represent a useful tool to increase genetic diversity in broodstock.

Liquid nitrogen cryopreservation is effective for long-term gene banking, but is only applicable for sperm preservation in most species. Cryopreserved sperm is useful for breeding programs and maintenance of genetic diversity, but the reconstitution of already extinct fish using cryopreserved sperm is difficult at present because sperm gives rise to inviable haploid progeny after induced androgenesis. Moreover, maternally inherited genotypes and cytoplasmic mitochondrial DNA are not preserved by sperm cryopreservation and thus never restored by androgenesis.

The loach (*Misgurnus anguillicaudatus*) is a fish widely distributed in Japan, and the species includes rare genotypes including natural diploid clones and polyploids (Arai, 2001, 2003). Previously, we cryopreserved loach sperm (Yasui et al., 2008, 2009) and reconstituted viable progeny by combining cryopreserved diploid sperm and artificially-induced androgenesis (Yasui et al., 2010). However, the resultant androgenotes exhibited low survival rates and were predominantly males due to unknown mechanisms (Fujimoto et al., 2010a). These androgenotes result presumably in reduced genetic diversity.

Technologies to produce germ-line chimeras were developed recently in loach (Nakagawa et al., 2002) by transplantation of primordial germ cells (PGC) (Saito et al., 2006; Fujimoto et al., 2006, 2010b). Targeted genotypes may be reconstituted by this method because the host should produce donor gametes in the germ-line chimeras (Yamaha et al., 2006). In a similar manner, cryopreservation of blastomeres followed by production of germ-line chimeras is considered a useful repository strategy. In the present study, we established a protocol for cryopreservation of blastomeres and subsequent production of germ-line chimeras in the loach.

MATERIALS AND METHODS

Embryo Management and Isolation of Blastomeres

This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals in Hokkaido University. Induced maturation of broodstock, dechoriation of eggs, and incubation of embryos followed procedures reported in previous studies (Fujimoto et al., 2004, 2007). Briefly, final maturation was induced with a single dose of hCG followed by gamete collection after 12 h at 27°C. Fertilization was achieved by the dry method in a Petri dish covered by a plastic film. Five min after fertilization, the water was removed from the dish and the remaining adhered eggs were fully covered by dechoriation medium (0.1% trypsin and 0.4% urea in Ringer's solution - 7.5 g/L NaCl, 0.2 g/L KCl, and 0.2 g/L CaCl₂, Yamaha et al., 1986). The dechorionated eggs were transferred to an agar-plated Petri dish filled with culture medium (Ringer solution with 1.6% albumen, 100 µg/mL streptomycin and 100 IU penicillin, Fujimoto et al., 2006).

At the blastula stage (512 to 1024 cells), blastoderms were removed mechanically using a fine needle. Control embryos were left intact. When controls reached 30% epiboly stage (Fujimoto et al., 2006), we carefully transferred 180 isolated blastoderms using a glass Pasteur pipette to a 2-mL microtube. The isolated blastoderms were rinsed in Ringer's solution to eliminate residues of yolk, and the supernatant was eliminated. Isolation of blastomeres was achieved by addition of 300 μ L of 0.25% sodium citrate (diluted in Ringer solution) following by gently pipetting using a wide-bore pipette tip.

Cryopreservation, Thawing, and Viability Assessment

Isolated blastomere suspension (in sodium citrate) was diluted 4-fold in a cryo-solution (1 part of blastomere suspension: 3 parts of cryo-solution) and mixed. This procedure resulted in a cell concentration of $356 \pm 75 \times 10^3$ cells/mL. The cryo-solution was prepared with Eagle's minimum essential medium (MEM) and dimethyl-sulfoxide (DMSO) at 0, 5, 10, 15, and 20% (vol/vol). The tube content was packed in 250- μ L cryogenic straws (IMV, L'Aigle, France), sealed with sealing powder and set in a programmable freezer (EYELA, MPF-1000, Tokyo, Japan) previously cooled at 5°C. As we used many treatments to optimize blastomere cryopreservation, the straw volume was reduced to 130 μ L by cutting, in order to reduce blastomere usage, but for transplantation procedures, we used the whole straw (250 μ L).

The samples were frozen using the following freezing procedure: hold at 5°C for 5 min followed by cooling at -0.75°C/min until -40°C and then directly plunging into liquid nitrogen for storage. Fresh (not cryopreserved) and cryopreserved blastomeres in MEM (without cryoprotectant) were used as control groups.

Samples were thawed in a water bath at 25°C for 10 s. The straw content was immediately transferred to a microtube with 200 µL of MEM containing 0.8 µL of propidium iodide (2.4 mM, Sperm viability kit, Invitrogen, Eugene, OR). After 5 min, the cells were placed in a hemocytometer and observed under a fluorescence microscope (Nikon Eclipse E800, Tokyo, Japan) with a specific filter for propidium iodide excitation (536 nm). Dead cells were identified by intense intracellular fluorescent staining (red), whereas live cells maintained their translucent-like appearance. The viability was assessed twice for each sample by counting the number of dead cells in a total of 250 to 300 cells.

Di-Methyl-Sulfoxide (DMSO) Concentration

Blastomeres were cryopreserved following the above-mentioned procedure, but we used different cryo-solutions containing MEM solution and DMSO concentrations at 0, 5, 10, 15, or 20% (vol/vol). Viability of post-thaw blastomeres was evaluated 3 d afterwards.

Glycerol Additions

Blastomeres were cryopreserved in Eagle's MEM solution with 10% DMSO and glycerol at 0, 1, 2, and 4% (vol/vol). Cryopreservation was performed as mentioned above. Viability of post-thaw blastomeres was evaluated 1 d afterwards.

External Cryoprotectants

Isolated blastomeres were cryopreserved in cryo-solution containing Eagle's MEM with 10% DMSO and containing the following cryoprotectants: bovine serum albumin (BSA) at 1 and 2% (wt/vol); sucrose at 1 and 2% (wt/vol); fetal bovine serum (FBS) at 5 and 10% (vol/vol). Cryopreservation was performed as mentioned above. Viability of post-thaw blastomeres was evaluated 62 d afterwards.

Transplantation of Blastomeres Immediately After Thawing

Artificial green fluorescence protein (*GFP*) mRNA was produced combining the 3'UTR of germ-line specific mRNA (*nos-1*) from the zebrafish (Köprunner *et al.*, 2001) using the mMESSAGE MACHINE kit (Ambion, Austin, TX). A new batch of fertilized eggs was dechorionated and each embryo was injected with zebrafish *GFP-nos1* 3'UTR mRNA (200ng/ μ L) containing 5% biotin-dextran fixable (Sigma, St. Louis, MO) for PGC labeling.

Blastomeres from these embryos were cryopreserved using our optimized protocol (see above). About 1 mo afterward, 3 straws (250 μ L) of blastomeres were thawed, transferred to a 1.5-mL microtube, and centrifuged at $800 \times g$ for 4 min. Most of the supernatant was removed and the cells were resuspended in 500 μ L of MEM solution with 100U/mL DNase (Invitrogen, Carlsbad, CA) and transferred to a 500- μ L tube. The cells were maintained for 30 min at 20°C for removal of the cryo-solution. After incubation, the tube was centrifuged again and half of the supernatant was removed. The tube was observed under the stereoscopic microscope and the blastomeres were collected from the bottom using a capillary glass micropipette connected to the microinjector apparatus (Figure 1). The micropipette had an inside diameter of 70 μ m and was beveled to a 30° angle.

For host embryos, another batch of eggs was obtained and the embryos were reared to the blastula stage. The collected blastomeres were injected at the lateral marginal part of host blastoderm and the transplanted embryos were maintained at 20°C.

Transplantation of GFP-Positive Blastomeres After Incubation

A new egg batch was obtained and blastomere labelling and cryopreservation were performed as mentioned above. Two straws were thawed and the content was placed in an agar-plated Petri dishes filled with culture medium. The Petri dishes were maintained at 20°C for 16 h. For host embryos, we obtained a new egg batch, each embryo was labeled by injection of zebrafish *DsRed-nos1* 3'UTR mRNA to identify host PGC. Donor blastomeres were observed under the fluorescence stereoscopic microscope and 1 to 3 GFP-positive cells were collected using a glass capillary micropipette (90- to 100-µm inside diameter). The cells were injected at the marginal part of each host blastoderm and cultured at 20°C.

Assessment of Induced Chimerism

Embryos were cultured at 20°C. Developmental stages (gastrulation, somite, and hatch) were determined following the criteria proposed by Fujimoto et al. (2006). Migration of PGC to the genital area was assessed in vivo by fluorescence microscopy: donor PGC exhibit GFP fluorescence and host PGC exhibit DsRed fluorescence. Four d postfertilization (dpf), the fish presenting donor PGC at the genital area were fixed in Bouin's fixative for 12 h, and then transferred to 80% ethanol. After paraffin embedding, serial 6-µm sections were collected and

the donor cells labeled with biotin were detected by biotin-streptavidin-horseradish peroxidase complex and 3,3'-diaminobenzidine substrate followed by hematoxylin and eosin counterstaining.

Statistics

Data are shown as mean $\pm$ SD. All experiments were performed in triplicate using different egg sources. Data were checked for normality using the Kolmogorov-Smirnov test and then compared using one-way ANOVA. Based on the number of replications, normality, and homogeneity of the data, the Tukey's multiple-range test ($P < 0.05$) was used in order to establish a comparison within treatments. The software Minitab version 15 for Windows was used to make the statistical analyses.

RESULTS

Cryopreservation of Blastomeres

Cryopreservation reduced the viability of the blastomeres (Figure 2). The addition of DMSO to MEM solution improved the post-thaw viability of blastomeres ($P < 0.0001$). Blastomere cryopreservation without DMSO resulted in a post-thaw viability of $1.8 \pm 0.8\%$. The DMSO additions at 5, 10, 15, and 20% improved the post-thaw viability to $31.1 \pm 13.7\%$, $44.1 \pm 11.5\%$, 32.0 ± 5.8 , and $36.9 \pm 12.9\%$, respectively. These results are lower when compared to

control unfrozen samples ($95.1 \pm 1.5\%$, $P < 0.0001$). Based on these results, DMSO concentration at 10% was selected for further cryopreservation experiments.

Glycerol supplementation significantly decreased ($P < 0.0001$) the post-thaw viability (Figure 3). In the absence of glycerol, post-thaw viability was $34.4 \pm 12.5\%$. Addition of glycerol at 1, 2, and 4% to the cryo-solution reduced post-thaw viability to $26.5 \pm 5.5\%$, $11.7 \pm 4.6\%$, and $4.7 \pm 1.2\%$, respectively. By comparison to fresh controls, cryopreservation reduced ($P < 0.0001$) viability.

The addition of external cryoprotectants did not significantly improve post-thaw viability when compared to control (cryopreserved with 10% DMSO) (Figure 4). Post-thaw viability after addition of external cryoprotectants ranged from $60.9 \pm 2.3\%$ (sucrose at 1%) to $67.0 \pm 2.1\%$ (FBS at 10%). These data did not significantly differ from control ($65.1 \pm 0.6\%$, $P < 0.0531$), in which the blastomeres were cryopreserved without any external cryoprotectants. However, post-thaw samples presented decreased viability when compared with control (fresh), in which the viability was $96.8 \pm 0.8\%$.

Production of Germ-Line Chimeras

Transplantation of blastomeres immediately after thawing gave very low survival rates, and most of the embryos died before gastrulation (Table 1). The number of germ-line chimeras also was very low (3 of 264 or 1.1%). Intact and dechorionated groups produced hatching rates about 70%, in which ~3% were abnormal.

Incubation of cryopreserved blastomeres for 16 h at 20°C prior to transplantation improved hatching rates (~90%) and the percentage of chimeras (16%; Table 2). Intact and

dechorionated treatments gave hatching rates around 80% with 5% abnormal embryos. Chimerism was confirmed in vivo by observation of host and donor PGC (Figure 5). Some donor PGC exhibited different migration routes than host PGC (Figures 5C and 5E). However, at hatching stages, donor and host PGC were localized to the genital area. Donor PGC migration was confirmed by histological detection of biotin-streptavidin-horseradish peroxidase at 4 d post transplantation (Figures 5G and 5H).

DISCUSSION

In previous studies with teleosts, blastomeres were cryopreserved using both slow- and rapid-freezing protocols. Using the slow cooling, the post-thaw viability was 54% for *Carassius auratus* (Kusuda et al., 2004), 59.3% for *Oncorhynchus keta* (Kusuda et al., 2002), 19.9% for *Sillago japonica*, 67.4% for *Odontesthes bonariensis*, 34.1% for *Oryzias latipes* (Strussmann et al., 1999), 70.2 to 84.8% for *Danio rerio* (Harvey, 1983; Lin et al., 2009); 96% for *Cyprinus carpio* (Calvi and Maisse, 1999), and 36 to 95% for *Oncorhynchus mykiss* (Nilson and Cloud, 1993; Calvi and Maisse, 1998; Kobayashi et al., 2003). The rapid-cooling method gave viability above 90% in the zebrafish (Cardona-Costa et al., 2009). Despite many protocols on blastomere cryopreservation mentioned above, very few attempts successfully produced germ-line chimeras using the cryopreserved samples. Cardona-Costa et al. (2009) failed to produce germ-line chimeras, although the viability of post-thaw blastomeres was above 90%. In *C. auratus*, Kusuda et al. (2002) used a similar procedure to the present study (transplantation of blastomeres immediately after thawing) but obtained a very low percentage of germ-line chimeras (~3%). An effective procedure was reported in zebrafish by Higaki et al. (2010), who succeeded in

production of germ-line chimeras using PGCs isolated from vitrified embryos (~8%). Kobayashi et al. (2007) produced germ-line chimeras from cryopreserved PGC and 5.6 to 12.1% of the transplanted fish successfully produced donor gametes in *Oncorhynchus mykiss*.

In our study, transplantation procedures played an important role in embryo survival as well as for successful formation of chimeras from cryopreserved blastomeres. The transplantation using blastomeres immediately post-thaw resulted in very low hatching rates and most embryos died shortly after injection. In such procedure, the volume of the transplanted cells was not the main cause of embryo mortality because germ-line chimeras were successfully produced in the same species using similar transplanted volume from donor blastoderms (Fujimoto et al., 2010b). However, in such method only the blastomeres were transplanted, differing from our procedure in which some volume of solution was also injected and may affect the embryo development. Another possible problem regards to the toxic residues from the cryo-solution, including DMSO and sodium citrate, and the presence of dead cells in post-thaw samples that may also disturb the development of transplanted embryos. Optimization of the cryopreservation methods examined viability of post-thaw blastomeres and did not test whether these surviving cells could develop in the host embryos. Later improvement in our procedure for cryopreservation (packaging, cooling protocols, cryoprotectants, and thawing conditions) and post-thaw management (centrifugeing, injection, and elimination of dead cells and toxic components) may be used to increase the production of germ-line chimeras by such method. The post-thaw management including washing and the usage of DNase used in this study is recommended because it improves the survival rate of the transplanted fish (unpublished data) but more adjustments may be necessary for successful production of germ-line chimeras.

Incubation of cryopreserved blastomeres prior to injection into the host allowed the blastomeres to reach the adequate period for expression of GFP-*nos1* mRNA in some of them. Transplantation of the GFP-positive blastomeres successfully produced 16% of germ-line chimeras. Such transplantation is interesting because the post-thaw recovery process probably eliminates, or dilutes, toxic components in the cryo-solution allowing for selection of viable, developing PGC.

In the case of fresh zebrafish (*Danio rerio*) samples from which PGC were isolated for transplantation, demonstrated that the yield of germ-line chimeras was around 5.7 to 32 %. (Kawakami et al., 2010) This range is similar to that observed in the present study (16.5%). The previous and present results showed that cryopreserved blastomeres could differentiate into PGC and they did not lose their migration ability to the genital ridges. It is possible that changes in post-thaw blastomere incubation periods may promote PGC differentiation and further improve our results.

A single PGC from the *Danio albolineatus* was successfully transplanted into *Danio rerio* and the host species produced heterospecific gametes (Saito et al., 2008). As discussed by many authors (Yoshizaki et al. 2003; Yamaha et al., 2003; Saito et al., 2008), successful production of chimeras may be facilitated when using closely-related species as hosts. In our case, in order to establish a protocol of production of germ-line chimeras using cryopreserved blastomeres, we performed intra-specific transplantation and the PGC were able to migrate and colonize to the genital ridges. This result suggested that our procedure may be effective for gamete production from donor PGC derived from cryopreserved blastomeres of loach.

In conclusion, we defined an effective protocol for cryopreservation of loach blastomeres. We also developed an improved method to produce germ-line chimeras from the cryopreserved

blastomeres integrating short-term culture and transplantation of differentiated PGC. These results are applicable for cryobanking and restoration of genetic resources from cryopreserved blastomeres.

LITERATURE CITED

Arai, K. 2001. Genetic improvement of aquaculture finfish species by chromosome manipulation techniques in Japan. *Aquaculture* 197:205-228.

Arai, K. 2003. Genetics of the loach *Misgurnus anguillicaudatus*: recent progress and perspectives. *Folia Biol.* 51:107-117.

Calvi, S. L., and G. Maisse. 1999. Cryopreservation of carp (*Cyprinus carpio*) blastomeres. *Aquatic Living Resour.* 12:71-74.

Calvi, S. L., and G. Maisse. 1998. Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) blastomeres: influence of embryo stage on postthaw survival rate. *Cryobiology* 36:255-262.

Cardona-Costa, J., M. Francisco-Simão, and F. Garcia-Ximénez. 2009. Can vitrified zebrafish blastomeres be used to obtain germ-line chimaeras? *CryoLetters* 30:422-428.

Fujimoto, T., T. Kataoka, S. Otani, T. Saito, T. Aita, E. Yamaha, and K. Arai. 2004. Embryonic stages from cleavage to gastrula in the loach *Misgurnus anguillicaudatus*. *Zool. Sci.* 21:747-755.

- 310 Fujimoto, T., T. Kataoka, S. Sakao, T. Saito, E. Yamaha, and K. Arai. 2006. Developmental
311 stages and germ cell lineage of the loach (*Misgurnus anguillicaudatus*). *Zool. Sci.* 23:977–
312 989.
- 313 Fujimoto, T., G.S. Yasui, M. Hayakawa, S. Sakao, E. Yamaha, and K. Arai. 2010a. Reproductive
314 capacity of neo-tetraploid loaches produced using diploid spermatozoa of a natural tetraploid
315 male. *Aquaculture* 308:S133-S139.
- 316 Fujimoto, T., T. Saito, S. Sakao, K. Arai, and E. Yamaha. 2010b. Developmental potential of
317 embryonic cells in a nucleocytoplasmic hybrid formed using a goldfish haploid nucleus and
318 loach egg cytoplasm. *Int. J. Dev. Biol.* 54:827-835.
- 319 Harvey, B. 1983. Cooling of embryonic cells, isolated blastoderms, and intact embryos of the
320 zebra fish *Brachydanio rerio* to -196°C . *Cryobiology* 20:440-447.
- 321 Hiemstra, S.J., T. Lende, H. van der Woelders. 2006. The potential of cryopreservation and
322 reproductive technologies for animal genetic resources conservation strategies . Pages 25-30
323 in *The Role of Biotechnology in Exploring and Protecting Agricultural Genetic Resources*. J.
324 Ruane, and A Sonnino, ed. FAO, Rome, Italy.
- 325 Higaki, S., Y. Eto, Y. Kawakami, E. Yamaha, N. Kagawa, M. Kuwayama, M. Nagano, S.
326 Katagiri, and Y. Takahashi. 2010. Production of fertile zebrafish (*Danio rerio*) possessing
327 germ cells (gametes) originated from primordial germ cells recovered from vitrified
328 embryos. *Reproduction* 139:733-740.
- 329 Kawakami, Y., R. Goto-Kazeto, T. Saito, T. Fujimoto, S. Higaki, Y. Takahashi, K. Arai, and E.
330 Yamaha. 2010. Generation of germ-line chimera zebrafish using primordial germ cells

- 331 isolated from cultured blastomeres and cryopreserved embryoids. *Int. J. Dev. Biol.* 54:1493-
332 1501.
- 333 Kobayashi, T., Y. Takeuchi, G. Yoshizaki, and T. Takeuchi. 2003. Cryopreservation of trout
334 primordial germ cells: a novel technique for preservation of fish genetic resources. *Fish*
335 *Physiol. Biochem.* 28:479–480.
- 336 Kobayashi, T., Y. Takeuchi, T. Takeuchi, and G. Yoshizaki. 2007. Generation of viable fish
337 from cryopreserved primordial germ cells. *Mol. Repr. Dev.* 74:207-213.
- 338 Köprunner, M., C. Thisse, B. Thisse, and E. Raz. 2001. A zebrafish *nanos*-related gene is
339 essential for the development of primordial germ cells. *Genes Dev.* 15:2877–2885.
- 340 Kusuda, S., T. Teranishi, and N. Koide. 2002. Cryopreservation of chum salmon blastomeres by
341 the straw method. *Cryobiology* 45:60-67.
- 342 Kusuda, S., T. Teranishi, N. Koide, T. Nagai, K. Arai, and E. Yamaha. 2004. Pluripotency of
343 cryopreserved blastomeres of the goldfish. *J. Exp. Zool.* 301A:131-138.
- 344 Lin, C., T. Zhang, and D.M. Rawson. 2009. Cryopreservation of zebrafish (*Danio rerio*)
345 blastomeres. *CryoLetters* 30:132-41.
- 346 Nakagawa, M., T. Kobayashi, and K. Ueno. 2002, Production of germline chimera in loach
347 (*Misgurnus anguillicaudatus*) and proposal of new method for preservation of endangered
348 fish species. *J. Exp. Zool.* 293:624–631.
- 349 Nilsson, E., and J.G. Cloud. 1993. Cryopreservation of rainbow trout (*Oncorhynchus mykiss*)
350 blastomeres. *Aquat. Living Resour.* 6:77-80.

- 351 Saito, T., T. Fujimoto, S. Maegawa, K. Inoue, K. Arai, and E. Yamaha. 2006. Visualization of
352 primordial germ cells *in vivo* using GFPnos1 3'UTR mRNA. *Int. J. Dev. Biol.* 50:691–699.
- 353 Saito, T., R. Goto-Kazeto, K. Arai, and E. Yamaha. 2008. Xenogenesis in teleost fish through
354 generation of germ-line chimeras by single primordial germ cell transplantation. *Biol. Rep.*
355 78:159-166.
- 356 Strussmann, C.A., H. Nakatsugawa, F. Takashima, M. Hasobe, T. Suzuki, and R. Takai. 1999.
357 Cryopreservation of isolated fish blastomeres: effects of cell stage, cryoprotectant
358 concentration, and cooling rate on postthawing survival. *Cryobiology* 39:252-261.
- 359 Yamaha, E., K. Usui, H. Onozato, and K. Hamada. 1986. A method for dechoriation in
360 goldfish *Carassius auratus*. *Bull. Jpn. Soc. Sci. Fish.* 52:1929-1934.
- 361 Yamaha, E., T. Saito, R. Goto-Kazeto, and K. Arai. 2006 Developmental biotechnology for
362 aquaculture, with special reference to surrogate production in teleost fishes. *J. Sea Res.* 58:8-
363 22.
- 364 Yasui, G.S., L. Arias-Rodriguez, T. Fujimoto, and K. Arai. 2009. A sperm cryopreservation
365 protocol for the loach *Misgurnus anguillicaudatus* and its applicability for other related
366 species. *Anim. Rep. Sci.* 116:135-145.
- 367 Yasui, G.S., L. Arias-Rodriguez, T. Fujimoto, and K. Arai. 2008. Simple and inexpensive
368 method for cryopreservation of fish sperm combining straw and powdered dry ice.
369 *CryoLetters* 29:383-390.

- 370 Yasui, G.S., T. Fujimoto, and K. Arai. 2010. Restoration of the loach, *Misgurnus*
371 *anguillicaudatus*, from cryopreserved diploid sperm and induced androgenesis. *Aquaculture*
372 308:S140-S144.
- 373 Yoshizaki, G., Y. Takeuchi, T. Kobayashi, and T. Takeuchi. 2003. Primordial germ cells: A
374 novel tool for fish bioengineering. *Fish Physiol. Biochem.* 28:453–457.

375 Tables

376

377 Table 1. Development of loach chimeric embryos by transplantation of cryopreserved blastomeres immediately after thawing. Number
378 within parenthesis indicates the number of embryos with primordial germ cells in the genital area.
379

Treatment	n	Unfertilized	Blastula	Gastrula	10 somite	Hatched embryos	
						Normal	Abnormal
Intact control	1,112	25.9 ± 11.3%	74.1 ± 11.3%	72.2 ± 12.9%	70.1 ± 14.0%	67.6 ± 13.2%	3.1 ± 1.4
Dechorionated	386	28.3 ± 11.6%	71.7 ± 11.6%	71.2 ± 12.3%	69.7 ± 14.2%	64.6 ± 12.0%	2.9 ± 1.2
Chimera	264	-	100.0 ± 0.0%	14.9 ± 5.9%	4.5 ± 3.5% (4)	1.4 ± 1.9% (1)	1.7 ± 1.4% (2)

380

381

382 Table 2. Development of loach chimeric embryos by transplantation of green fluorescence protein-positive blastomeres. Number
 383 within parenthesis indicates the number of embryos with primordial germ cells in the genital ridge.
 384

Treatment	n	Unfertilized	Blastula	Gastrula	10 somite	Hatched embryos	
						Normal	Abnormal
Intact control	653	$9.8 \pm 8.5\%$	$90.2 \pm 8.5\%$	$87.8 \pm 8.1 \%$	$86.6 \pm 8.9 \%$	$81.71 \pm 13.1\%$	$4.86 \pm 6.2\%$
Dechorionated	472	$9.7 \pm 7.9\%$	$90.3 \pm 7.9 \%$	$89.3 \pm 9.3 \%$	$84.3 \pm 15.5 \%$	$79.2 \pm 15.4 \%$	$5.15 \pm 3.5\%$
Chimera	206	-	$100.0 \pm 0.0 \%$	$92.1 \pm 7.4 \%$	$90.1 \pm 10.9 \%$	$80.2 \pm 13.6\% (28)$	$9.5 \pm 2.5\% (5)$

385

Figure captions:

Figure 1. Apparatus used to collect cryopreserved blastomeres, prepared using an oil microinjector (Celltram Vario, Eppendorf, Hamburg, Germany) and a 1-mL syringe barrel. The pipette holder was attached to a micromanipulator (M-152, Narishige, Tokyo, Japan). Blastomere suspension was centrifuged in a 0.5-mL microtube, and the cells were collected from the bottom using an inverted capillary micropipette connected to the apparatus. Arrows indicates the flow of oil pumping for cell collection.

Figure 2. Viability of cryopreserved blastomeres using cryo-solutions containing increasing concentrations of dimethyl sulfoxide in Eagle's minimum essential medium. Percentage of live and dead cells was assessed by propidium iodide fluorescent staining. Columns without a common letter differ as determined by the Tukey test ($P < 0.05$).

Figure 3. Viability of cryopreserved blastomeres using cryo-solutions containing 10% dimethyl sulfoxide in Eagle's minimum essential medium and increasing glycerol additions. Percentage of live and dead cells was assessed by propidium iodide fluorescent staining. Columns without a common letter differ as determined by the Tukey test ($P < 0.05$).

Figure 4. Viability of cryopreserved blastomeres using cryo-solutions containing 10% dimethyl sulfoxide in Eagle's minimum essential medium and various external cryoprotectants and concentrations. Control fresh refers to non-cryopreserved blastomeres and in control the

blastomeres were cryopreserved without external cryoprotectant (10% dimethyl sulfoxide only). Percentage of live and dead cells was assessed by propidium iodide fluorescent staining. Columns without a common letter differ as determined by the Tukey test ($P < 0.05$).

Figure 5. Embryos transplanted using cryopreserved, thawed and incubated blastomeres. Left images show embryos at 12-somite stage (A, C, E). Right images show hatched larvae (B, D, and F). Host (C and D) and donor primordial germ cells (PGC) (E and F) were labeled by injection of DsRed and GFP-*nos1* 3'UTR mRNA, respectively, and observed by fluorescence. Chimeric embryo observed under a normal light (A and B). Histological sections of transplanted PGC by biotin-streptavidin-horseradish peroxidase complex and 3,3'-diaminobenzidine substrate and then stained with hematoxylin and eosin (G and H). Arrows indicate PGC. No = notocord; p = pronephric duct, and y = yolk.

421 Figure 1.

422

423

424 Figure 2.

425

426

427

428 Figure 3.

429

430

431 Figure 4.

432

433