Generation of juvenile rainbow trout derived from cryopreserved whole ovaries by intraperitoneal transplantation of ovarian germ cells.

Generation of juvenile rainbow trout derived from cryopreserved whole ovaries by intraperitoneal transplantation of ovarian germ cells

Seungki Lee[a,b], Naoto Katayama[a], Goro Yoshizaki[a,*]

[a]Department of Marine Biosciences, Tokyo University of Marine Science and Technology, Tokyo 108-8477, Japan

[b]Biological and Genetic Resources Assessment Division, National Institute of Biological Resources, Incheon 404-708, Korea

[*]Corresponding author

Tel.: +81-3-5463-0558. Fax: +81-3-5463-0558. Email: goro@kaiyodai.ac.jp

Abstract

Cryopreservation of fish sperm offers the practical applications in the selective breeding and biodiversity conservation. However, because of the lack of cryopreservation methods for fish eggs and embryos, maternally inherited cytoplasmic compartments cannot be successfully preserved. We previously developed an alternative method to derive functional eggs and sperm from cryopreserved whole testis by transplanting testicular cells into female and male recipients. However, if target fish had ovaries, the previous method employing male-derived germ cells would be ineffective. Here, we aimed to generate functional gametes from cryopreserved whole ovaries by transplanting ovarian germ cells into peritoneal cavity of sterile hatchlings. Cryopreservation conditions for rainbow trout ovaries (1.0 M DMSO, 0.1 M trehalose, and 10% egg yolk) were optimized by testing several different cryoprotective agents. Ovarian germ cells from thawed ovaries were intraperitoneally transplanted into allogeneic triploid hatchlings. Transplanted germ cells migrated toward and were incorporated into recipient gonads, where they underwent gametogenesis. Transplantation efficiency of ovarian germ cells remained stable after cryopreservation period up to 1,185 days. Although all triploid recipients that did not undergo transplantation were functionally sterile, 5 of 25 female recipients and 7 of 25 male recipients reached sexual maturity at 2.5 years post-transplantation. Inseminating the resultant eggs and sperm generated viable offspring displaying the donor characteristics of orange body color, green fluorescence, and chromosome numbers. This method is thus a breakthrough tool for the conservation of endangered fish species that are crucial to cryopreserve the genetic resources of female fish.

1. Introduction

Many fish species face a high risk of extinction; thus, preservation of fish fertility is vital for conserving the biodiversity of threatened fish species. Cryopreservation is highly effective for the long-term preservation of fish genetic resources [1]. However, because of the lack of cryopreservation techniques for fish eggs and embryos [1,2], maternally inherited cytoplasmic compartments cannot be successfully preserved [2,3]. Accordingly, although fish genetic resources have been maintained by rearing viable individuals, this approach is vulnerable to pathogen outbreaks, natural disasters, and the reduced fitness of individual fish within natural habitats [4,5].

We recently showed alternative methods for deriving functional eggs and sperm from cryopreserved whole testis [6] or frozen male fish [7] by transplanting testicular cells into female and male recipients. However, with gonadally female target fish, the methods employing male-derived germ cells [6,7] would be ineffective. For example, overfishing of protogynous fish that can change their gonadal sex from female to male [8] can cause biased sex ratios with large number of females [9,10]; thus, female fish are more valid targets for preserving their genetic resources. Furthermore, with endangered fish species having sex-determination mechanisms with female heterogamety (e.g., ZZ/ZW sex-determination system) [8, 11–12], the female-specific W chromosome would not be preserved by the previously established methods [6,7]. Therefore, a new method for preserving and restoring female genetic resources is needed to protect endangered fish species.

Recently, female germline stem cells were identified in the ovaries of medaka fish [13], and the oogonial stem cells were found capable of making intrinsic sperm-egg fate decisions [14]. Methods for cryopreservation [15,16] and transplantation [17–19] of ovarian germ cells have been developed for several fish species. However, no reports have described the derivation of fish eggs and sperm from cryopreserved ovarian germ cells. We previously developed a surrogate broodstock technology [17] to

produce donor-derived eggs and sperm by transplanting ovarian germ cells into the peritoneal cavity of sterile trout hatchlings. Here we investigated the generation of functional eggs and sperm following cryopreservation of ovarian germ cells and subsequent germ cell transplantation.

2. Materials and methods

2.1. Fish

Rainbow trout (*Oncorhynchus mykiss*) were maintained at the Oizumi Research and Training Station of Tokyo University of Marine Science and Technology (Yamanashi, Japan). Orange-colored (heterozygous, OR/WT) [20] p*vasa-Gfp* transgenic (hemizygous, *vasa-Gfp*/–) [21,22] rainbow trout females (chromosome number, 2n = 60,XX) [6] were used as donors. Wild-type triploid rainbow trout (WT/WT/WT and –/–/–; chromosome number, 3n = 88) [6,23] were germ cell recipients. All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Tokyo University of Marine Science and Technology.

2.2. Ovary cryopreservation

Immature ovaries (ovary weight, 0.016 ± 0.004 g; gonad-somatic index, 0.089% ± 0.005%) were isolated from 9-month-old transgenic rainbow trout (standard length, 11.1 ± 0.2 cm) whose ovarian germ cells, including oogonia and early stage oocytes, specifically expressed green fluorescent protein (GFP) [17]. Freshly isolated ovaries were transferred into 1.2-mL cryotubes (TPP, Switzerland) containing 500 μL cryomedium (pH 7.8) that comprised permeating cryoprotectant agent (CPA; propylene glycol, ethylene glycol, dimethyl sulfoxide, or glycerol at 1.0, 1.3, or 1.6 M), nonpermeating CPA (0.1 M D-glucose or 0.1 M D-[+]-trehalose dihydrate and 1.5% [wt/vol] BSA, 1.5% [vol/vol] FBS, 10% [vol/vol] fresh egg yolk, or 1.5% [wt/vol] skim milk powder), and 35.2% (vol/vol) extender. The

cryotubes were maintained on ice for 60 min and cooled at −1 °C/min for 90 min using a Bicell plastic freezing container (Nihon Freezer, Japan) located in a −80 °C freezer before immersion in liquid nitrogen. After cryopreservation in liquid-phase nitrogen, cryotubes were quickly thawed in a 10 °C water bath and rehydrated [6]. Extender was formulated as described [6]. All cryopreservation reagents were purchased from Sigma-Aldrich.

2.3. Viability assessment of germ cells

Fresh and frozen ovaries were dissociated into single cells [24], and the resultant cell suspensions were filtered through a 20-μm-pore nylon screen (Tokyo Screen Company, Japan). Because the total numbers of GFP (+) germ cells did not significantly differ between both ovaries of rainbow trout (n = 4, $P < 0.05$), the numbers of GFP (+) germ cells in frozen (1, 73, 273, 450, 642, and 1,185 days) and fresh ovaries were compared to determine the viability of cryopreserved GFP (+) germ cells (n = 12–24). Survival of GFP (+) germ cells was assessed using a Guava PCA-96 flow cytometry system (Millipore) and trypan blue (TB)-exclusion assay. The viability of GFP (+) germ cells was calculated as: viability (%) = ([number of GFP-positive and TB-negative cells in frozen ovary]/[number of GFP-positive cells in fresh ovary]) × 100.

2.4. Germ cell transplantation

Approximately 20−30 nL cell suspension, containing ~10,000 GFP (+) cells, was intraperitoneally transplanted into 33-day-old hatchlings. At 20, 50, and 193 days post-transplantation (pt), recipients were dissected and their gonads were observed under fluorescent microscopes (BX51-34FL and MVX10, Olympus). The ratios of recipients with GFP (+) donor cells in their gonads at 20 and 50 days pt and the number of incorporated GFP (+) cells at 20 days pt were recorded (n = 101–133).

Colonization and proliferation efficiencies of GFP (+) germ cells were calculated as: colonization rate (%) = ([number of fish incorporating GFP-positive cells at 20 days pt]/[number of fish observed]) × 100; proliferation rate (%) = ([number of fish proliferating GFP-positive cells at 50 days pt]/[number of fish observed]) × 100.

2.5. Progeny test

Recipients were reared until maturity. To determine the generation of sperm from frozen GFP (+) donor cells, total genomic DNA was extracted from the semen of male recipients and analyzed by PCR, using *Gfp*-specific primers [25]. At 2.5 years pt, eggs from female recipients were inseminated with milt from male recipients of the same experimental group. To determine the production of F1 offspring by gametes derived from frozen donor cells, donor phenotypes (orange-color, *vasa-Gfp*, and ovary) were examined in the F1 offspring. To determine the phenotypic sex of the F1 offspring, the middle portions of the ovaries isolated from randomly selected F1 juveniles (n = 22) were fixed in Bouin solution, paraffin-embedded, serially sectioned at 5-μm thickness, and stained with hematoxylin and eosin (H&E). The genotypic sex of F1 juveniles was further examined by PCR. Total genomic DNA was extracted from the fins of randomly selected F1 juveniles (n = 13) and analyzed by PCR with *sdY*-specific primers [26]. To confirm the cytogenetic normality of the F1 juveniles, the DNA contents and karyotype analyses of the donor rainbow trout (n = 10), recipients (n = 12), and randomly selected F1 juveniles (n = 38) were conducted [6].

2.6. Statistical analysis

Data are shown as the mean ± standard error of the mean from three independent experiments. Statistical significance was determined by one-way analysis of variance followed by Tukey's multiple-

comparisons test. Student's t-test was used for comparisons between two groups. $P < 0.05$ indicated statistical significance.

3. Results

3.1. Viability of cryopreserved ovarian germ cells

When slowly cooled ovaries were cryopreserved for 1 day, significantly higher viability was found with GFP (+) germ cells cryopreserved with cryomedium containing 1.3 M dimethyl sulfoxide (DMSO) instead of 1.3 M propylene glycol (PG) or 1.3 M glycerol (GLY) (Fig. 1A). Using ovaries cryopreserved in cryomedium containing ethylene glycol (EG) or DMSO at 1.0, 1.3, or 1.6 M concentrations, we found that GFP (+) germ cells cryopreserved in 1.0 M DMSO tended to exhibit the highest survival rate (Fig. 1B). Next, the effects of nonpermeating CPAs dissolved in cryomedium containing 1.0 M DMSO on GFP (+) germ cell survival were assessed. The viability of GFP (+) germ cells was significantly higher in ovaries cryopreserved in cryomedium containing 0.1 M trehalose and 1.5% (wt/vol) BSA (Fig. 1C). Finally, the effects of 1.5% (wt/vol) BSA, 10% (vol/vol) egg yolk, or 1.5% (wt/vol) skim milk dissolved in cryomedium containing 1.0 M DMSO and 0.1 M trehalose on GFP (+) germ cell survival were compared. GFP (+) germ cells with the highest survival rate (72.9% ± 6.2%) were obtained from ovaries whose cryomedium contained 10% (vol/vol) egg yolk (Fig. 1D). Ovaries were cryopreserved for 1–1,185 days to assess the long-term viability of GFP (+) germ cells within ovaries frozen in the optimized cryomedium containing 1.0 M DMSO, 0.1 M trehalose, and 10% (vol/vol) egg yolk. The viability of GFP (+) germ cells obtained from the cryopreserved ovaries did not change significantly from 1–1,185 days (Fig. 1E).

3.2. Transplantation of cryopreserved ovarian germ cells

To determine whether ovarian germ cells (Fig. 1K) recovered from long-term cryopreserved ovaries (Fig. 1I,J) maintained stem cell potency [17,27], the transplantation efficiencies of ovarian germ cells frozen for 1 or 1,185 days were compared with those of freshly prepared germ cells. Recipients were dissected at 20, 50, and 193 days pt, and the behavior of GFP (+) donor cells were examined. At 20 days pt, GFP (+) germ cells frozen for 1,185 days were incorporated into the gonads in 73/102 recipients examined (Fig. 1L,P). The mean number of GFP (+) germ cells incorporated into the gonads was 2.0 ± 0.2 in the 1,185-day-frozen group (Fig. 1L,Q). The 1,185-day-frozen cells began to proliferate rapidly within the gonads in 64/112 recipients at 50 days pt (Fig. 1M,R) and resumed oogenesis and spermatogenesis in 24/52 female recipients (Fig. 1N,N′) and 29/58 male recipients (Fig. 1O,O′), respectively, at 193 days pt. Colonization (Fig. 1P) and proliferation efficiency (Fig. 1R) of donor germ cells within recipient gonads, and the germ cell-incorporation rate (Fig. 1Q) did not differ significantly with different freezing periods versus freshly prepared controls.

3.3 Eggs and sperm derived from cryopreserved ovarian germ cells

Although all triploid females that had not received transplants were completely sterile, 5/25 (20%) female CP8 recipients (CP8, recipients that received germ cells cryopreserved for 8 months) reached sexual maturity at 2.5 years pt (Table 1). A similar maturation rate was observed with fresh control recipients receiving freshly prepared germ cells (Table 1). The number of eggs ovulated from CP8 recipients (1,575 ± 242) did not significantly differ from those ovulated from fresh control recipients and wild-type diploid trout (Fig. 2A).

All triploid males that had not received transplants were sterile, other than 3 exceptional males that produced small amounts of aneuploid sperm incapable of generating normal hatchlings (Fig. 2B, Table 1). In contrast, 4/29 (13.8%) and 7/25 (28.0%) male CP8 recipients reached sexual maturity at

1.5 and 2.5 years pt, respectively (Table 1). The numbers of sperm ($0.8 \pm 0.2 \times 10^{10}$ and $1.9 \pm 0.3 \times 10^{10}$ at 1.5 and 2.5 years pt, respectively) obtained from CP8 males did not significantly differ from those of fresh control recipients and wild-type diploid trout (Fig. 2B). The genetic background of sperm obtained from the CP8 and fresh male recipients at 2.5 years pt was examined by PCR with *Gfp*-specific primers [25]. All milt from the recipients was positive for the presence of the *Gfp* gene (Fig. 2C).

3.4. Production of donor-derived rainbow trout

At 2.5 years pt, eggs ovulated from female recipients were inseminated with milt from male recipients. The fertilization (98.4% ± 0.7%), eyed (90.2% ± 1.6%), hatching (86.6% ± 1.2%), and swim-up rates (83.4% ± 1.9%) of fertilized eggs from the CP8 recipients did not differ significantly from those of eggs produced by fresh control recipients and wild-type diploid trout (Fig. 2D). When the donor phenotypes of cryopreserved ovaries (OR and *vasa*-*Gfp*) were successfully transmitted to F1 generation, approximately 75% of F1 juveniles were expected to exhibit a donor phenotype, following Mendelian inheritance. In the F1 juveniles produced by CP8 recipients, the percentages of orange-colored (76.6% ± 1.3%) and *vasa*-*Gfp* (+) (74.1% ± 1.8%) fish approached 75% (Fig. 2E–I, Supplementary Table 1). Furthermore, when donor germ cells carrying XX sex chromosomes were successfully transmitted to the F1 generation, the F1 juveniles should be 100% female. Microscopic observations of ovaries isolated from F1 juveniles produced by CP8 recipients (n = 1,500; Fig. 2J,K, Supplementary Table 1), histological analyses (n = 22; Fig. 2L), and PCR analyses with *sdY*-specific primers (n = 13; Fig. 2M) indicated that the F1 juveniles were all female. The same results were observed with fresh control recipients receiving freshly prepared germ cells (Supplementary Table 1). Analyses of the DNA content for 38 F1 juveniles produced by CP8 recipients revealed that all F1

juveniles were normal diploid (Fig. 2P) and none showed signs of aneuploidy or triploidy (Fig. 2O). All F1 juveniles possessed 60 chromosomes with 104 arm numbers (Fig. 2S), which was identical to these characteristics in the donor trout (Fig. 2Q), but clearly differed from those of the triploid recipients (Fig. 2R).

4. Discussion

The results of this study demonstrate that juvenile rainbow trout could be derived from cryopreserved whole ovaries after intraperitoneal transplantation of ovarian germ cells. The viability and transplantation efficiencies of frozen ovarian germ cells did not change for cryopreservation periods up to 1,185 days. The long-term-frozen (8 months) ovarian germ cells possessed high capacity for differentiating into eggs and sperm in the ovaries and testes of recipients. Insemination of the resultant eggs and sperm generated normal rainbow trout that displayed the donor-derived characteristics of orange body color, green fluorescence, XX ovaries, and chromosome numbers. Because all triploid trout not receiving transplants were functionally sterile, we conclude that the F1 offspring obtained from triploid transplant recipients were all derived from frozen donor ovaries.

Previously, we established a technique for generating functional eggs and sperm from cryopreserved testicular germ cells in rainbow trout [6] and applied this technique to conserving endangered fish species [28]. However, this method [6,28] cannot be directly applied to female fishes because it utilizes male germ cells. Here, we developed a new method that can be effectively applied for cryopreserving the genetic resources of female fish. For example, with protogynous fish such as Atlantic goliath grouper (*Epinephelus itajara*) or with female heterogametic fish such as European eel (*Anguilla anguilla*) and Siberian sturgeon (*Acipenser baerii*), where cryopreserving female germ cells is crucial, the technique established in this study would provide a key solution. Additionally, because

methods to cryopreserve eggs and embryos in amphibians utilizing female heterogamety for sex determination have yet to be developed [29], our results also have significant implications for conserving their biodiversity.

Here, we demonstrated that all F1 offspring from frozen XX ovarian germ cells were XX females. Previously, [30] we showed that recipients transplanted with rainbow trout XY male germ cells produced X sperm, X eggs, Y sperm, and Y eggs, and that YY supermales were produced in the F1 generation after random fertilization. When YY supermales, which do not normally appear in natural environments, are released into the species' original habitat, this could result in the extinction of species [31]. Thus, when germ cell-transplantation techniques are used to conserve species that employ male heterogamety for sex determination, the appearance of YY supermales can be a significant disadvantage. When X eggs (produced by female recipients transplanted with XX female germ cells) and X or Y sperm (produced by male recipients transplanted with X or Y male germ cells) are inseminated, a general 1:1 sex ratio can be induced without the appearance of YY supermales in the F1 generation [30]. Therefore, our new method can be significantly used with endangered fish species that employ male heterogamety for sex determination.

Here, we optimized the CPA conditions (1.0 M DMSO, 0.1 M trehalose, and 10% egg yolk) for cryopreserving the ovarian germ cells from rainbow trout, which were nearly identical to those optimized for cryopreserving the testicular germ cells from the same species (1.3 M DMSO, 0.1 M trehalose, and 10% egg yolk) [6]. Pšenička et al. [16] recently reported that 1.5 M ethylene glycol, 50 mM glucose, and 0.5% BSA is the best cryoprotectant for both ovarian and testicular germ cells in Siberian sturgeon. Additional studies using different fish species are required to determine whether the conditions of cryoprotectant agents differ between ovarian germ cells and testicular germ cells.

The method of whole-ovary cryopreservation established here is simple and does not require

241 complex laboratory equipment such as programmable freezers, enabling its use under field conditions.
242 Therefore, using this method would substantially contribute to the conservation of endangered fish
243 species that are crucial to cryopreserve the genetic resources of female fish. This is the first report of
244 successfully generating functional gametes from cryopreserved female germ cells in any fish species.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research (KAKENHI) on Innovative Areas, “Mechanisms regulating gamete formation in animals” and the Ocean Resource Use Promotion Technology Development Program conducted by MEXT.

References

[1] P. Mazur, S.P. Leibo, G.E. Seidel, Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions, Biol. Reprod. 78 (2008) 2–12.

[2] S. Martínez-Páramo, Á. Horváth, C. Labbé, et al., Cryobanking of aquatic species, Aquaculture (2016) doi: 10.1016/j.aquaculture.2016.05.042.

[3] G. Yoshizaki, K. Fujinuma, Y. Iwasaki, et al., Spermatogonial transplantation in fish: a novel method for the preservation of genetic resources, Comp. Biochem. Physiol. Part D Genomics Proteomics 6 (2011) 55–61.

[4] H. Araki, B. Cooper, M.S. Blouin, Genetic effects of captive breeding cause a rapid, cumulative fitness decline in the wild, Science 318 (2007) 100–103.

[5] M.R. Christie, M.L. Marine, R.A. French, et al., Genetic adaptation to captivity can occur in a single generation, Proc. Natl. Acad. Sci. USA 109 (2012) 238–242.

[6] S. Lee, Y. Iwasaki, S. Shikina, et al., Generation of functional eggs and sperm from cryopreserved whole testes, Proc. Natl. Acad. Sci. USA 110 (2013) 1640–1645.

[7] S. Lee, S. Seki, N. Katayama, et al., Production of viable trout offspring derived from frozen whole fish, Sci. Rep. 5 (2015) 16045.

[8] R.H. Devlin, Y. Nagahama, Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences, Aquaculture 208 (2002) 191–364.

[9] S.S. Heppell, S.A. Heppell, F.C. Coleman, et al., Models to compare management options for a protogynous fish, Ecol. Appl. 16 (2006) 238–249.

[10] E.E. Easter, J.W. White, Spatial management for protogynous sex-changing fishes: a general framework for coastal systems, Mar. Ecol. Prog. Ser. 543 (2016) 223–240.

[11] D. Fopp-Bayat, Meiotic gynogenesis revealed not homogametic female sex determination system in Siberian sturgeon (*Acipenser baeri* Brandt), Aquaculture 305 (2010) 174–177.

[12] B. Geffroy, A. Bardonnet, Sex differentiation and sex determination in eels: consequences for management, Fish Fish. 17 (2016) 375–398.

[13] S. Nakamura, K. Kobayashi, T. Nishimura, et al., Identification of germline stem cells in the ovary of the teleost medaka, Science 328 (2010) 1561–1563.

[14] T. Nishimura, T. Sato, Y. Yamamoto, et al., *foxl3* is a germ cell–intrinsic factor involved in sperm-egg fate decision in medaka, Science 349 (2015) 328–331.

[15] L.S. Marques, A. Bos-Mikich, L.C. Godoy, et al., Viability of zebrafish (*Danio rerio*) ovarian follicles after vitrification in a metal container, Cryobiology 71 (2015) 367–373.

[16] M. Pšenička, T. Saito, M. Rodina, et al., Cryopreservation of early stage Siberian sturgeon *Acipenser baerii* germ cells, comparison of whole tissue and dissociated cells, Cryobiology 72 (2016) 119–122.

[17] G. Yoshizaki, M. Ichikawa, M. Hayashi, et al., Sexual plasticity of ovarian germ cells in rainbow trout, Development 137 (2010) 1227–1230.

[18] Z. Csenki, A. Zaucker, B. Kovács, et al., Intraovarian transplantation of stage I-II follicles results in viable zebrafish embryos, Int. J. Dev. Biol. 54 (2010) 585–589.

[19] T.T. Wong, A. Tesfamichael, P. Collodi, Production of zebrafish offspring from cultured female germline stem cells, PLoS One 8 (2013) e62660.

[20] S. Boonanuntanasarn, G. Yoshizaki, K. Iwai, et al., Molecular cloning, gene expression in albino mutants and gene knockdown studies of tyrosinase mRNA in rainbow trout, Pigment Cell Res. 17 (2004) 413–421.

[21] G. Yoshizaki, S. Sakatani, H. Tominaga, et al., Cloning and characterization of a *vasa*-like gene

in rainbow trout and its expression in the germ cell lineage, Mol. Reprod. Dev. 55 (2000) 364–371.

[22] Y. Takeuchi, G. Yoshizaki, T. Kobayashi, et al., Mass isolation of primordial germ cells from transgenic rainbow trout carrying the green fluorescent protein gene driven by the *vasa* gene promoter, Biol. Reprod. 67 (2002) 1087–1092.

[23] T. Okutsu, S. Shikina, M. Kanno, et al., Production of trout offspring from triploid salmon parents, Science 317 (2007) 1517–1517.

[24] T. Okutsu, K. Suzuki, Y. Takeuchi, et al., Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish, Proc. Natl. Acad. Sci. USA 103 (2006) 2725–2729.

[25] Y. Takeuchi, G. Yoshizaki, T. Takeuchi, Biotechnology: surrogate broodstock produces salmonids, Nature 430 (2014) 629–630.

[26] A. Yano, R. Guyomard, B. Nicol, et al., An immune-related gene evolved into the master sex-determining gene in rainbow trout, *Oncorhynchus mykiss*, Curr. Biol. 22 (2012) 1423–1428.

[27] S.M. Lacerda, G.M Costa, L.R. de França, Biology and identity of fish spermatogonial stem cell, Gen. Comp. Endocrinol. 207 (2014) 56–65.

[28] S. Lee, G. Yoshizaki, Successful cryopreservation of spermatogonia in critically endangered Manchurian trout (*Brachymystax lenok*), Cryobiology 72 (2016) 165–168.

[29] J. Clulow, S. Clulow, Cryopreservation and other assisted reproductive technologies for the conservation of threatened amphibians and reptiles: bringing the ARTs up to speed, Reprod. Fertil. Dev. 28 (2016) 1116–1132.

[30] T. Okutsu, S. Shikina, T. Sakamoto, et al., Successful production of functional Y eggs derived from spermatogonia transplanted into female recipients and subsequent production of YY

supermales in rainbow trout, *Oncorhynchus mykiss*, Aquaculture 446 (2015) 298–302.

[31] J.B. Gutierrez, J.L. Teem, A model describing the effect of sex-reversed YY fish in an established wild population: the use of a Trojan Y chromosome to cause extinction of an introduced exotic species, J. Theor. Biol. 241 (2006) 333–341.

Figure legends

Fig. 1. Transplantation of cryopreserved ovarian germ cells. (**A**) Viability of germ cells in frozen-thawed ovaries with medium containing 1.3 M propylene glycol (PG), ethylene glycol (EG), dimethyl sulfoxide (DMSO) or glycerol (GLY) (*n* = 14). (**B**) Viability of germ cells with medium containing EG or DMSO at various concentrations (1.0, 1.3, or 1.6 M; *n* = 12). (**C**) Viability of germ cells with medium containing 0.1 M glucose or trehalose cryoprotectants with BSA or FBS (*n* = 12). (**D**) Viability of germ cells with BSA, egg yolk, or skim milk with medium containing 1.0 M DMSO and 0.1 M trehalose (*n* = 12). (**E**) Viability of germ cells within ovaries cryopreserved for 1–1,185 days. No significant differences in cell viability were observed following the different cryopreservation periods (*n* = 12–24). (**F–H**) Freshly prepared trout ovary in a bright-field (**F**) and fluorescence view (**G**), and dissociated ovarian cells (**H**). (**I–K**) Trout ovary cryopreserved for 1,185 days in a bright-field (**I**) and fluorescence view (**J**), and dissociated ovarian cells (**K**). (**L**) GFP (+) germ cells cryopreserved for 1,185 days were incorporated into recipient gonads at 20 days post-transplantation. (**M–O**) Incorporated GFP (+) germ cells began proliferating (arrowheads) at 50 days post-transplantation (**M**) and resumed gametogenesis in female (**N, N′**) and male recipients (**O,O′**) at 193 days post-transplantation. (**P–R**) The percentage of recipients that contained donor germ cells within their gonads (**P**), the number of germ cells incorporated into the recipient gonads (**Q**), and the percentage of recipients having proliferated donor germ cells (**R**) were not significantly different between germ cells cryopreserved for 1 (CP1) or 1,185 days (CP1185), or freshly prepared germ cells (fresh) (*n* = 101–133). The data shown are the mean ± standard error of the mean derived from three independent experiments. Scale bars, 1 mm (**F,G,I,J**), 50 μm (**H,K–M**), 100 μm (**N–O′**).

Fig. 2. Generation of trout derived from cryopreserved ovarian germ cells. (**A**) Numbers of eggs ovulated by recipients that received ovarian germ cells frozen for 8 months (CP8), recipients that received freshly prepared germ cells (fresh), and wild-type (WT) diploid trout at 2.5 years post-transplantation. (**B**) Numbers of sperm produced by CP8 recipients, fresh recipients, WT diploid trout, and WT triploid trout (Triploid). The numbers within each bar indicate the number of mature fish (**A,B**). (**C**) PCR analyses of recipient milts with *Gfp*-specific primers. Lanes: M: molecular-weight marker, No. 1–8: milt obtained from recipients, P: *Gfp*-plasmid, WT: milt from WT diploid trout, DW: distilled water. (**D**) Developmental performances of fertilized eggs obtained from CP8 recipients (white bars), fresh recipients (gray bars), and WT diploid trout (black bars). (**E–I**) Approximately 75% of F1 juveniles derived from CP8 recipients displayed the donor-derived phenotypes of orange body color (**E**) and GFP-positive germ cells (**F,H**). The boxed area in (**E**) shows both phenotypic colors of the F1 progeny. Ovaries of orange-colored transgenic (**F**) and non-transgenic (**G**) F1 juveniles, and ovaries of WT transgenic (**H**) and non-transgenic (**I**) F1 juveniles are shown. (**J,K**) Transgenic (**J**) and non-transgenic (**K**) ovaries at high magnification. (**L**) Histological analysis of selected ovaries from F1 juveniles produced by CP8 recipients confirmed the presence of an ovary (n = 22). (**M**) PCR analysis of F1 juvenile fins with *sdY*-specific primers (top) or *β-actin*-specific primers (bottom). Lanes: M: molecular-weight marker, No. 1–13: fins of F1 juveniles derived from CP8 recipients, WT-M: WT male trout, WT-F: WT female trout, DW: distilled water. (**N–P**) Analyses of DNA content revealed that all F1 juveniles were normal diploid (**P**) as the DNA content in donor trout (**N**), but clearly different from the DNA content of CP8 recipients (**O**) (n = 10–38). (**Q–S**) Karyotype of donor trout (2n = 60) (**Q**), CP8 triploid recipients (3n = 88) (**R**), and F1 juveniles (2n = 60) (**S**) (n = 10–38). Scale bars, 50 μm (**J–L**), 10 μm (**Q–S**).

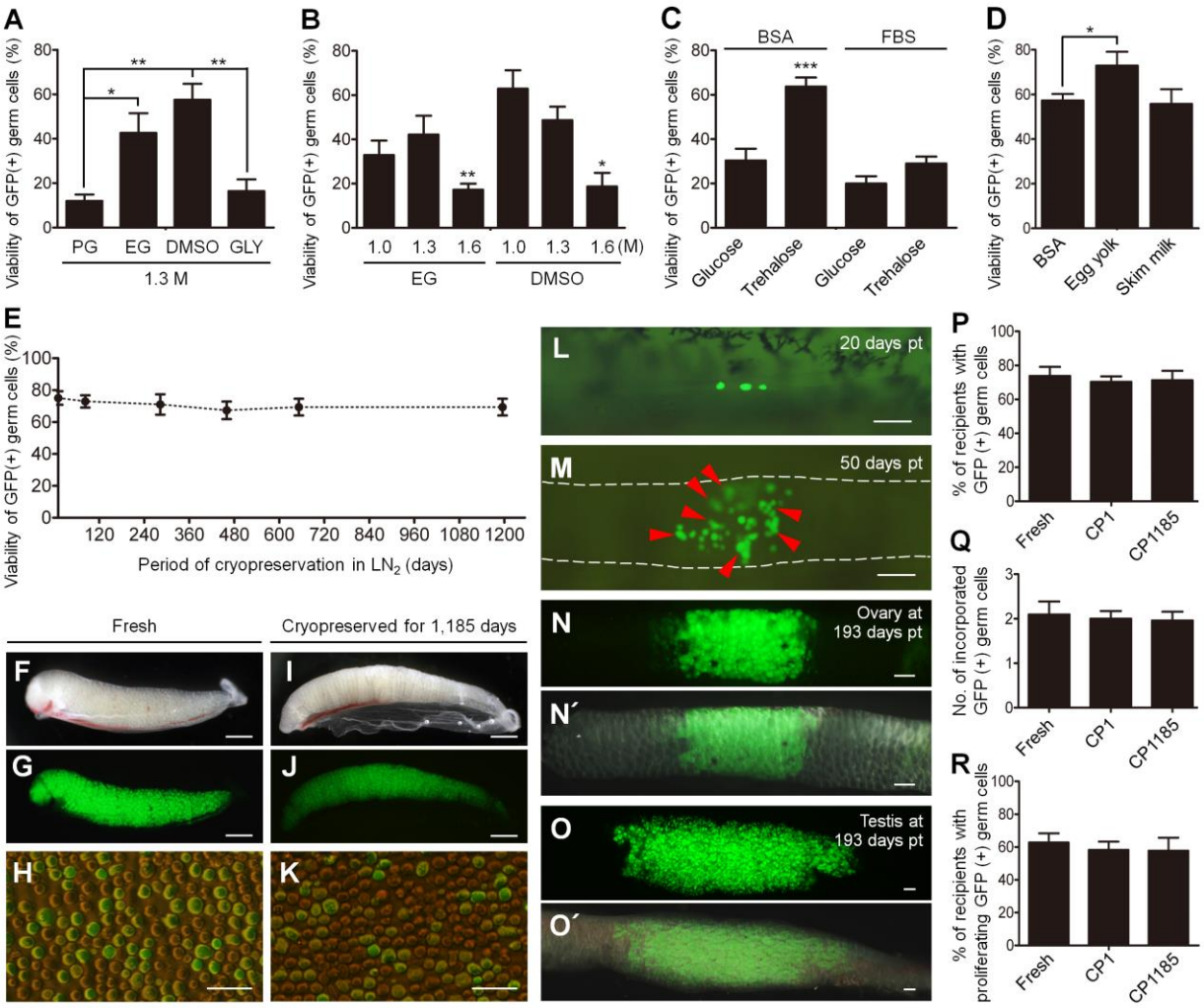
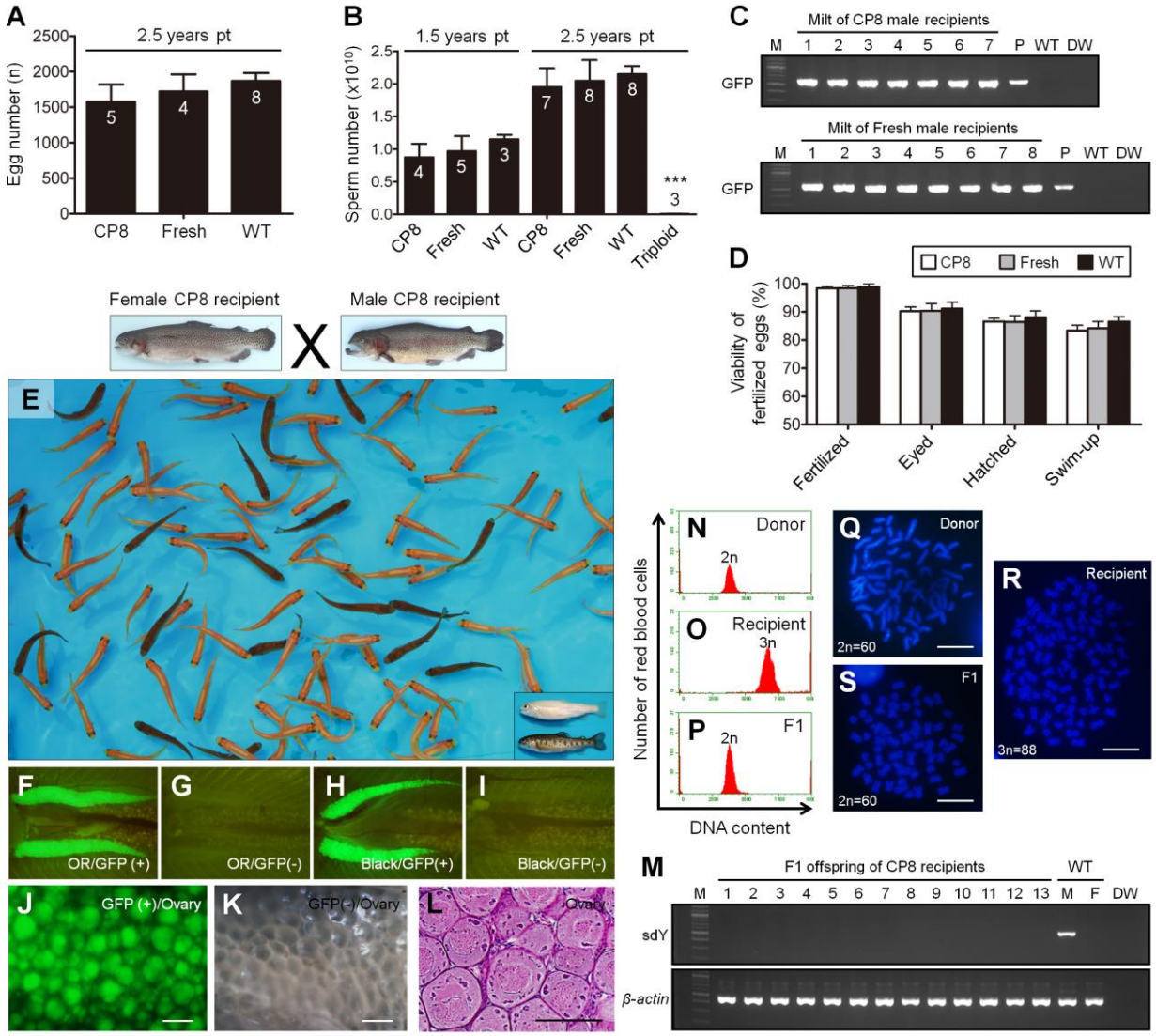

A
B
C
D
Viability of GFP(+) germ cells (%)
PG
EG
DMSO
GLY
1.3 M
1.0
1.3
1.6
1.6 (M)
BSA
FBS
Glucose
Trehalose
Egg yolk
Skim milk
E
Period of cryopreservation in LN2 (days)
Fresh
Cryopreserved for 1,185 days
L
20 days pt
M
50 days pt
N
Ovary at 193 days pt
N´
O
Testis at 193 days pt
O´
P
% of recipients with GFP (+) germ cells
Q
No. of incorporated GFP (+) germ cells
R
% of recipients with proliferating GFP (+) germ cells
CP1
CP1185

Figure 1.



373

374 **Figure 2.**

375

Table 1. Maturation of triploid recipients following transplantation of frozen-thawed ovarian germ cells.

Group	Cryopreservation period (months)	No. of fish	No. mature/surviving fish (%)			
			1.5 years post-transplantation		2.5 years post-transplantation	
			Female	Male	Female	Male
CP8[a]	8	70	0/32 (0.0)	4/29 (13.8)	5/25 (20.0)	7/25 (28.0)
Fresh[b]	-	70	0/26 (0.0)	5/33 (15.2)	4/22 (18.2)	8/26 (30.8)
Wild-type[c]	-	32	0/13 (0.0)	3/12 (25.0)	8/9 (88.9)	8/8 (100)
Triploid[d]	-	50	0/22 (0.0)	0/20 (0.0)	0/18 (0.0)	3/17 (17.6)*

[a]Triploid recipients received ovarian germ cells taken from whole ovaries cryopreserved for 8 months.

[b]Triploid recipients received freshly prepared ovarian germ cells.

[c]Wild-type diploid trout that did not undergo transplantation.

[d]Wild-type triploid trout that did not undergo transplantation.

[*]Triploid males produced a low number of aneuploid sperm.

376

377