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Spermatogonial Transplantation in Fish: Production of Trout Offspring from Salmon Parents

Tomoyuki Okutsu, Yutaka Takeuchi and Goro Yoshizaki*

Department of Marine Biosciences
Tokyo University of Marine Science and Technology
4-5-7 Konan, Minato
Tokyo 108-8477, Japan

*E-mail: goro@kaiyodai.ac.jp

Many salmonid species have recently become at risk of extinction. For fish species where eggs cannot be cryopreserved, establishment of techniques to preserve genetic resources other than egg and embryo cryopreservation is imperative. In the present study, spermatogonia from male trout were transplanted into the peritoneal cavity of newly hatched sterile triploid salmon. Transplanted trout spermatogonia colonized the gonads of sterile salmon recipients. In male recipients, transplanted spermatogonia underwent spermatogenesis. Further, in female recipients, transplanted spermatogonia underwent oogenesis. At 2 years after transplantation, sterile salmon recipients only produced donor-derived trout sperm and eggs. Fertilization with the sperm and eggs obtained from the triploid salmon recipients, only the donor-derived trout offspring was successfully produced. Combined with cryopreservation of spermatogonia, the present technique would make it possible to preserve fish genetic resources and to revive extinct species when necessary.

KEYWORDS spermatogonia; germ cell transplantation; vasa; surrogate broodstock

1. Introduction

In recent years, researchers have reported that if environmental destruction and commercial fishing continue at the current pace, the entire fishery resource will be depleted in about 40 years (Worm *et al*. 2006). Over the last decade, numbers have decline for many salmonid species, with some species becoming extinct while others have become endangered (U.S. Fish & Wildlife Service 2008). Particularly in North America, many salmonids, such as certain stocks of Chinook salmon (*Oncorhynchus tshawytscha*), Gila trout (*O. gilae*), and bull trout (*Salvelinus*

confluentus) are endangered. Many salmonid species have been placed on the endangered species list (U.S. Fish & Wildlife Service 2008) and urgent measures appear necessary. Although the first choice for saving endangered species is to preserve and improve the environment, some species and local populations are likely to disappear before habitats can be restored. Urgent backup technologies, therefore, need to be developed while preserving and improving the environment (Myers *et al*. 2003). At present, one backup technique is to raise endangered species in captivity, but this involves risks such as facility accidents, outbreaks of infectious disease, and the inability of farmed fish to adapt to the original natural environments. Cryopreservation of gametes and early embryos is a backup technique that does not carry such risks. In principle, semi-permanent preservation is possible, and this technique for genetic resource preservation is less expensive and less risky than raising fish in captivity. However, cryopreservation of fish eggs has not been accomplished due to the large size, low permeability of chorion, and their high fat content (Chao and Liao 2001). As an alternative method of genetic resource preservation for fish, we have been developing surrogate broodstock technologies. Surrogate broodstock technologies refer to techniques where immature germ cells from a target fish species are transplanted into a related species that is easy to raise and mature, so that the related species can produce sperm and eggs of the target species (Yoshizaki *et al*. 2002, 2003; Okutsu *et al*. 2006a). Immature germ cells, such as primordial germ cells (PGCs) and spermatogonia, are sufficiently small to be easily cryopreserved (Kobayashi *et al*. 2007). By cryopreserving the germ cells of an endangered species, even if that species goes extinct, the species can be revived by transplanting thawed PGCs or spermatogonia into a related species. Recently, we have succeeded in obtaining functional rainbow trout (*O. mykiss*) sperm from masu salmon (*O. masou*) by transplanting trout PGCs into the peritoneal cavity of newly hatched salmon and allowing the recipient salmon to mature (Takeuchi *et al*. 2004). However, the following issues must be resolved for surrogate broodstock technology using PGCs. First, fish PGCs can only be collected within a period of a few weeks before and after hatching, and since PGCs are not great in quantity, gathering sufficient numbers for transplantation is difficult. Second, when transplanting PGCs, most spermatozoa produced by recipients are of recipient origin, and few donor-derived spermatozoa are produced. Third, production of donor-derived functional eggs in xenogeneic recipients has not been possible by the method with trout PGCs. Okutsu *et al*. reported that a great quantity of spermatogonia could be obtained from the testes of male fish in all developmental stages and that spermatogonia could differentiate into both eggs and sperm after transplantation into newly hatched embryos (Okutsu *et al*. 2006b). The present study thus aimed to produce trout-derived sperm and eggs by transplanting spermatogonia into salmon recipients. Further, by using sterile triploid salmon as recipients, production of all donor-derived offspring was attempted.

2. Salmon Recipients Produce Donor-Derived Trout Sperm and Eggs Following Interspecies Transplantation of Spermatogonia

To verify whether the fish spermatogonial transplantation technology (Okutsu *et al*. 2006b) is valid for interspecies application, trout spermatogonia were microinjected into the peritoneal cavities of 90 salmon recipients by the method previously established in our laboratory (Takeuchi *et al*. 2003; Okutsu *et al*. 2006b). In this experiment, p*vasa-Gfp* (Yoshizaki *et al*. 2000; Takeuchi *et al*. 2002) hemizygous (p*vasa-Gfp*/–), dominant orange-colored mutant (Boonanuntanasarn *et al*. 2004) heterozygous (OR/wt), adult male rainbow trout and wild-type masu salmon

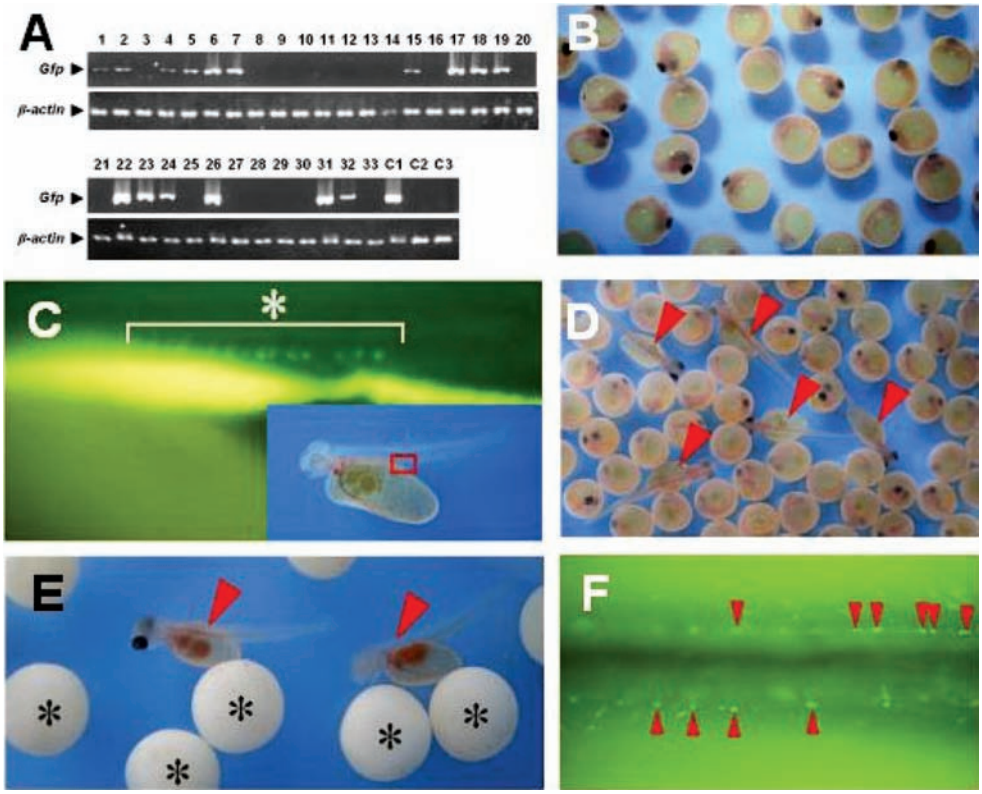


Fig. 1. Germline transmission of donor-derived haplotype to F1 progeny of diploid salmon recipients. **A**) PCR analysis of recipient milt samples with *Gfp*-specific primers. Lanes were labeled as follows: C1, positive control (milt of p*vasa-Gfp* trout); C2, negative control (milt of wild-type trout); C3, negative control (milt of wild-type salmon); 1–33, milt samples from diploid salmon male recipients. Positive results for the *Gfp* gene were seen in 16 milt samples. **B**) Hybrid obtained from wild-type male salmon and wild-type female trout at 34 dpf. No hybrids started hatching. **C**) Lateral view of orange-colored offspring that hatched before 34 dpf (inset), and a magnified image of the frame indicated in the inset showing *Gfp*-expressing PGCs (asterisk). The donor-derived haplotype was confirmed by *Gfp* expression in PGCs. **D**) F1 offspring obtained from a diploid salmon male recipient and wild-type female trout at 34 dpf. Most offspring had not hatched at this stage, although some had (arrowheads). **E**) F1 offspring obtained from diploid salmon female recipient and wild-type male trout at 34 dpf. Most offspring died before 34 dpf (asterisks), whereas some with normal morphology had hatched (arrowheads). **F**) Ventral view of the peritoneal cavity of F1 orange-colored offspring in E. The donor-derived haplotype was confirmed by *Gfp* expression in PGCs (arrowheads).

were used as donors and recipients, respectively. The masu salmon is a Pacific salmon found only in East Asia, whereas the rainbow trout is native to North America. These two species have been phylogenetically separated for at least 8 million years (McKay *et al*. 1996). Furthermore, only lethal hybrids can be produced from these two species. At 2 years after transplantation, 33 mature male and 38 mature female recipients were successfully obtained. Of these, 16 males were identified to produce donor-derived spermatozoa following PCR analysis with *Gfp*-specific primers (Fig. 1A). Progeny tests

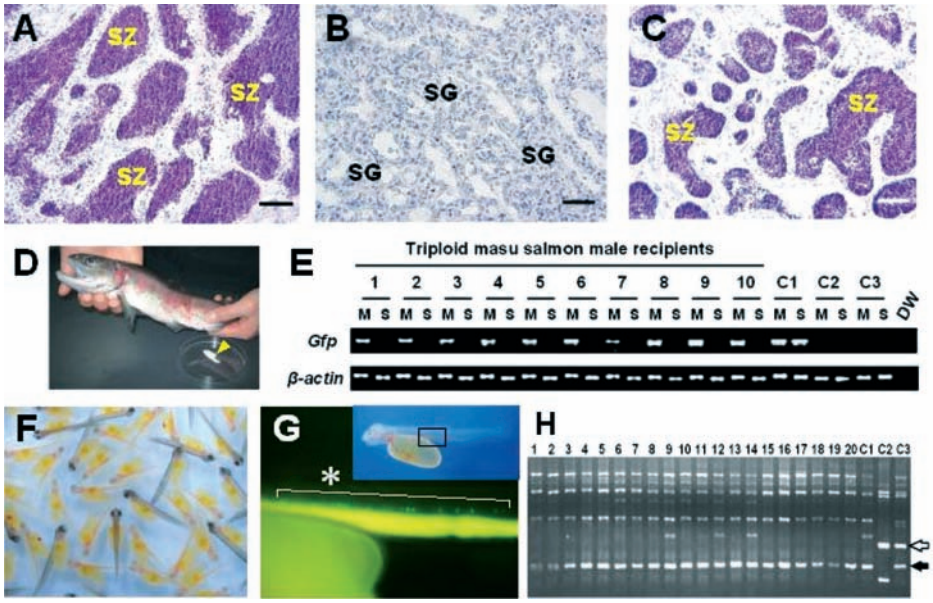
Table 1. Appearance rate of donor-derived rainbow trout among F1 generation of diploid masu salmon male recipients

Male recipient		Recipient-derived hybrids (*n*)	Donor-derived rainbow trout (*n*)	Total F1 offspring (*n*)	Donor-derived/Total (%)
5		1097	2	1099	0.2
6		970	34	1004	3.4
6	–2nd	406	12	418	2.9
18		960	26	986	2.6
18	–2nd	198	116	314	36.9
19		4	42	46	91.3
20		766	4	770	0.5
24		720	176	896	19.6
27		600	26	626	4.2
27	–2nd	757	116	873	13.3
32		412	140	552	25.4
33		769	8	777	1.0

were performed using 16 *Gfp*-positive male salmon with wild-type female trout. If male salmon produced donor-derived trout sperm, the next generation would consist of hybrids from recipient-derived salmon sperm and trout eggs and pure trout from donor-derived trout sperm and trout eggs. When raised at a water temperature of 10°C, almost all trout eggs hatched before 34 days post-fertilization (dpf), but no trout egg/salmon sperm hybrid eggs hatched before 34 dpf (Fig. 1B). As a result, in the following generation, nine recipients (9/33, 27.3%) produced offspring that hatched prior to 34 dpf and retained orange-colored body and Gfp-labeled PGCs (Figs. 1C, D), clearly identifying these offspring as donor-derived trout. Mean germline transmission rate for the donor-derived haplotype was 18.9% (range, 0.2–91.3%) (Table 1). When mating female salmon recipients and male wild-type trout in a progeny test, one of 38 female recipients produced two normal hatchlings (hatched within 34 dpf) among 324 hatchlings (0.6%) (Fig. 1E). One hatchling displayed orange-colored body and Gfp-labeled PGCs (Figs. 1E, F). In addition, salmon egg/trout sperm hybrids did not develop normally and could not hatch (Fig. 1E; Table 4). These normal hatchlings with orange-colored body and Gfp-labeled PGCs were thus clearly donor-derived trout. In this manner, the fish spermatogonial transplantation technology was shown to be valid for interspecies application, and we succeeded in obtaining donor-derived sperm and eggs from salmon recipients. Comparing the present method using spermatogonia to the existing method using PGCs as donor cells (Takeuchi *et al*. 2004), the ratio of xenogeneic recipients producing donor-derived sperm was about 10-fold higher (27.3% vs. 2.7%, respectively) and germline transmission rate was about 50-fold higher (18.9% vs. 0.4%, respectively).

3. Sterile Triploid Salmon Male Recipients Produce Only Trout-Offspring

Spermatogonia were collected from donor trout displaying the above-mentioned characteristics (p*vasa-Gfp*/–; OR/wt) and transplanted into 100 newly hatched triploid salmon recipients. Just prior to the spawning season at 2 years after transplantation, testes were extracted from some male recipients and analyzed histologically. The results showed that while the testes of triploid salmon in the control group (no transplanta-



Fig, 2. Germline transmission of donor-derived haplotype to F1 progeny of sterile triploid salmon male recipients. **A–C**) HE-stained section of testis. Wild-type diploid salmon (A), intact triploid salmon (B) and triploid salmon recipient that received spermatogonial transplantation (C). Normal spermatogenesis (spermatozoa, SZ) was seen in the testes of triploid recipients in addition to wild-type diploid salmon, whereas intact triploid salmon testis retained only undifferentiated spermatogonia (indicated as SG). Bar scales: 50 μm. **D**) The milt was successfully obtained (arrowhead) from a triploid salmon recipient. **E**) PCR analysis of milt (M) and soma (S) of triploid recipients with *Gfp*-specific primers. Lanes were labeled as follows: C1, p*vasa-Gfp* trout; C2, wild-type trout; C3, wild-type salmon; 1–10, triploid salmon male recipients. *Gfp* genes were detected in all milt of recipients not in soma. **F**) F1 offspring obtained from triploid salmon male recipient at 34 dpf. Orange-colored and wild-type hatchlings were obtained in similar proportions. **G**) Lateral view of orange-colored offspring that hatched before 34 dpf (inset), and a magnified image of the frame indicated in the inset. *Gfp* was expressed in PGCs (asterisk). **H**) RAPD analysis of F1 offspring. Lanes are labeled as follows: C1, wild-type trout; C2, wild-type salmon; C3, hybrid between trout and salmon; 1–20, F1 offspring of triploid salmon recipients. All offspring retained trout-specific bands (black arrow), not salmon-specific bands (white arrow). Although 20 out of 50 are shown here, the remaining samples showed identical banding patterns as lanes 1 to 20.

tion) were immature and filled with mostly spermatogonia (Fig. 2B), testes of recipient salmon contained spermatozoa just like normal salmon (Figs. 2A, C). At 2 years after transplantation, 10 of the 29 male triploid salmon recipients (34%) exhibited normal secondary sexual features and produced milt containing a large quantity of spermatozoa (Fig. 2D). Conversely, none of the triploid salmon without transplantation produced sperm.

PCR detected the *Gfp* gene specific to donor trout in the genomic DNA of sperm collected from all 10 recipients (Fig. 2E). Next, milts collected from these 10 recipients were used to fertilize wild-type trout eggs. F1 fertilized eggs all hatched before 34 dpf (Fig. 2F). In the F1 generation, the ratio of orange to wild-type colors was approximately 1:1 (302:304) (Fig. 2F; Table 2). In addition, an average of 49.3% possessed Gfp-labeled PGCs (Fig. 2G; Table 2). At this

Table 2. Appearance rate of F1 offspring with donor-derived haplotype among F1 generation of triploid salmon male recipients

Triploid masu salmon male recipient	Age of recipients (years)	Wild-type	Albino	Albino/Total (%)	Gfp+	Gfp−	Gfp+/Total (%)
#1	2	282	238	45.8	39	40	49.4
#2	2	388	423	52.2	21	15	58.3
#3	2	345	366	51.5	NE*	NE*	NE*
#4	2	321	342	51.6	41	40	50.6
#5	2	350	321	47.8	NE*	NE*	NE*
#6	2	275	301	52.3	41	51	44.6
#7	2	263	265	50.2	48	60	44.4
#8	2	284	255	47.3	42	34	55.3
#9	2	258	257	49.9	47	52	47.5
#10	2	251	272	52.0	44	56	44.0
Wild type**	2	452	0	0.0	0	61	0.0
#1	3	107	108	50.2	NE*	NE*	NE*
#3	3	113	84	42.6	NE*	NE*	NE*
#4	3	75	89	54.3	NE*	NE*	NE*
Wild type**	2	191	0	0.0	NE*	NE*	NE*

*NE: not examined; **Wild type: wild-type rainbow trout

time, development rate in the F1 generation was comparable to that in control wild-type trout (Table 3). Five F1 fish were collected from each of the 10 recipients, and these 50 fish were subjected to random amplified polymorphic DNA (RAPD) analysis for species determination. All 50 fish displayed the same DNA fingerprinting pattern as trout (Fig. 2H). These findings proved that the generation following male triploid salmon recipients produced only donor-derived trout (Okutsu *et al*. 2007). Furthermore, to measure the contamination rate of salmon cells in milt produced by triploid salmon recipients, semi-quantitative PCR analysis was performed using a masu salmon growth hormone gene-specific primer (Nagano *et al*. 1994). Contamination rates of salmon genomic DNA were low, ranging from 0.0–1.3%. While low levels of contamination by salmon cells were confirmed, the generation after male triploid salmon recipients exhibited development rates comparable to those of normal trout (Table 3), and all 50 F1 fish subjected to RAPD exhibited the same fingerprinting pattern as normal trout, suggesting that salmon genomic DNA originated from somatic cells including blood cells in milt. These findings clarified that the 10 triploid salmon recipients only produced donor-derived trout sperm. It has been known that male triploid salmonids occasionally produce aneuploid sperm (Carrasco *et al*. 1998), but the resulting next generation cannot survive. Furthermore, DNA content was measured in 67 F1 fish, and the DNA content for these F1 fish was identical to that for normal diploid trout, suggesting that triploid salmon recipients only produced donor-derived trout sperm (Fig. 3). In addition, triploid salmon recipients only produced large quantities of donor-derived sperm at 2 and 3 years old to spawn only donor-derived trout (Tables 2 and 3). These findings suggest that trout spermatogonia functioned as spermatogonial stem cells in the testes of triploid xenogeneic recipients. The cause of sterility in triploid male salmonids has not been clarified, but the present study is the first to indicate that the

Table 3. Development of F1 trout derived from triploid masu salmon male recipients at 2 and 3 years old

Triploid masu salmon male recipient	Age of recipients (years)	Total eggs (*n*)	Fartilized (%)	Eyed (%)	Hatched (%)	Swim up (%)
#1	2	697	73.3	74.6	73.5	73.2
#2	2	817	100.0	99.3	99.3	98.5
#3	2	715	100.0	99.4	99.2	98.9
#4	2	668	100.0	99.3	99.3	98.7
#5	2	676	100.0	99.3	89.3	88.8
#6	2	578	100.0	99.7	99.7	98.6
#7	2	532	100.0	99.2	98.5	97.4
#8	2	550	100.0	98.0	90.7	90.2
#9	2	523	100.0	98.5	98.5	98.1
#10	2	533	100.0	98.1	94.4	94.4
Wild type*	2	831	100.0	99.0	97.8	96.5
#1	3	238	100.0	99.6	98.7	96.6
#3	3	210	100.0	93.8	91.0	88.6
#4	3	168	100.0	97.6	97.6	96.4
Wild type*	2	201	100.0	95.0	93.5	89.6

*Wild type: wild-type rainbow trout

cause of sterility in male triploid salmonids is the germ cells themselves, and the gonadal microenvironment of male triploid salmonids is capable of maintaining the "stemness" of spermatogonial stem cells and has the proper endocrine system for spermatogenesis.

4. Successful Production of Only Trout Offspring from Sterile Salmon Parents

Some female 17-month-old triploid salmon recipients were dissected to examine the ovaries, and in four of eight recipients, the ovary contained donor-specific green-fluorescent oocytes (Fig. 4A). At this age, these oocytes had proceeded to vitellogenesis just like oocytes produced by normal diploid salmon (Figs. 4A, B). At the same age, ovaries of triploid salmon that had not received spermatogonia were underdeveloped, containing no vitellogenic oocytes (Fig. 4C). When recipients reached 2 years, five of the 50 female triploid salmon recipients ovulated from 38 to 213 eggs (Table 4). In order to develop the present surrogate broodstock technology as a practical technique, successful production of donor-derived offspring from xenogeneic recipients is essential. Therefore, these eggs were fertilized using milt harvested from male triploid salmon recipients that had been shown to produce only trout sperm, in an attempt to produce donor-derived next-generation trout from

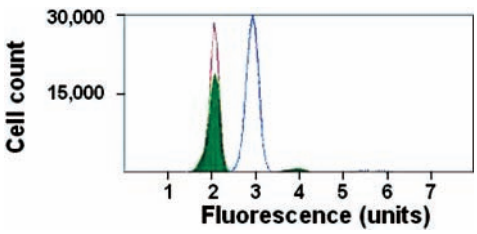


Fig. 3. Ploidy analysis of F1 offspring obtained from triploid salmon male recipient. Red line indicates wild-type diploid trout. Blue line indicates triploid salmon. Green indicates F1 offspring. DNA amount (ploidy) for F1 offspring was identical to that for wild-type diploid trout.

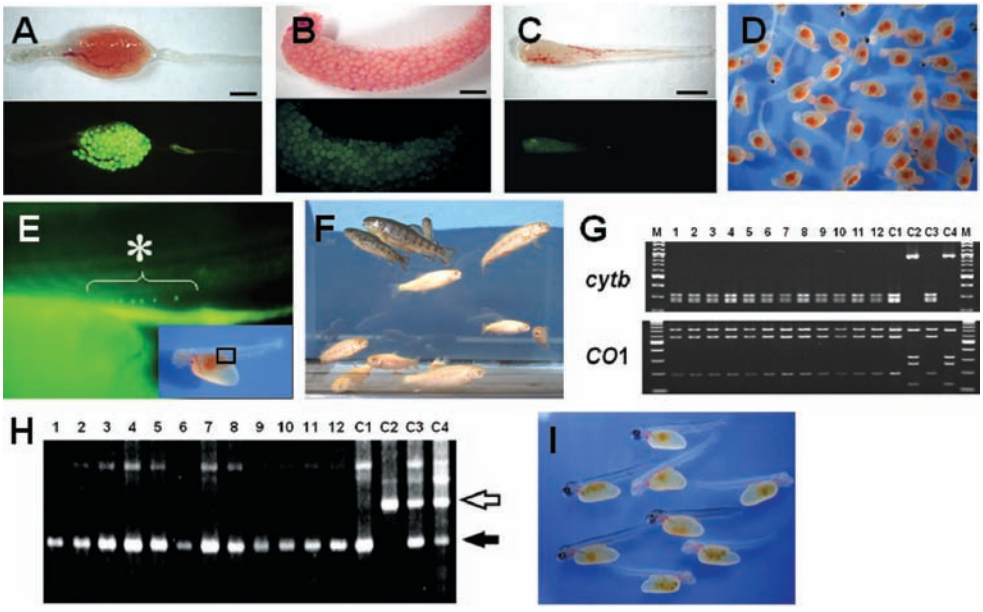


Fig. 4. Development of donor derived-oocytes and F1 offspring generated from surrogate parents. **A–C**) Oocyte colony derived from donor trout-spermatogonia in the ovary of triploid salmon recipient 17 months after transplantation (A), and ovaries of diploid salmon (B) and intact triploid salmon (C) at the same age as the recipient. Lower panels, fluorescent view. Bar scales: 5 mm. **D**) F1 offspring obtained from surrogate salmon parents at 34 dpf. **E**) Lateral view of orange-colored offspring (inset), with a highly magnified image of a frame in inset. *Gfp* was expressed in PGCs (asterisk). **F**) Juveniles generated from surrogate salmon parents at 6 months old. **G**) RFLP analysis of mitochondrial genes, *cytb* and *CO1*. Lanes were labeled as follows: M, MW marker; 1–12, PCR products obtained from template DNA of F1 offspring produced by surrogate salmon parents; C1, wild-type trout; C2, wild-type salmon; C3, hybrid between trout female and salmon male; C4, hybrid between salmon female and trout male. All F1 offspring showed digestion patterns identical to trout. **H**) RAPD of nuclear genome of F1 offspring produced by surrogate salmon parents. Lanes are labeled in the same way as in G. All offspring retained a trout-specific band (black arrow), not salmon-specific band (white arrow). **I**) F2 offspring obtained from F1 male.

xenogeneic male and female recipients. Although development rate of the next generation varied markedly from one female brood-stock to the next, hatching and swimming-up rates reached 89.5% and 57.9%, respectively, in some recipients (Table 4). All F1 hatchlings obtained from these triploid salmon parents hatched before 34 dpf, as in normal trout, and the ratios of orange-colored trout to wild-type trout and of p*vasa-Gfp*(+) and p*vasa-Gfp*(–) were both about 3:1 in the F1 generation (Figs. 4D, E). These findings show that the inheritance of OR/wt and p*vasa-Gfp*/– was Mendelian, and in other words, the F1 generation was generated from donor-derived sperm and eggs. Swim-up juveniles also developed normally (Fig. 4F). Next, restriction fragment length polymorphism (RFLP) analysis was performed to ascertain the mitochondrial origin of the F1 generation obtained from triploid salmon parents, clarifying that all 18 F1 fish carried trout mitochondria (Fig. 4G). These findings clarify that female triploid salmon recipients that received trout spermatogonia only produced normal functional donor-derived trout eggs. In addition, RAPD analysis of nuclear DNA showed that the DNA fingerprinting

Table 4. Development of F1 generation derived from xenogeneic surrogate parents

Total masu salmon female recipient	Eggs (*n*)	Eyed eggs (*n*, %)		Hatched embryos (*n*, %)		Swim-up juveniles (*n*, %)	
#1	178	0	(0.0)	0	(0.0)	0	(0.0)
#2	38	36	(94.7)	34	(89.5)	22	(57.9)
#3	154	4	(2.6)	3	(1.9)	3	(1.9)
#4	312	14	(4.5)	7	(2.2)	2	(0.6)
#5	310	155	(50.0)	155	(50.0)	65	(21.0)

Table 5. Development of F2 offspring produced using milt of F1 males of xenogeneic surrogate salmon parents

F1 male derived from surrogate parent	Egg numbers used for insemination	Fertilized (%)	Eyed (%)	Hatched (%)
F1-1	262	97.2	91.2	91.2
F1-2	212	100.0	92.0	91.0
F1-3	225	97.2	83.6	82.2
F1-4	242	97.2	97.5	96.7
F1-5	209	100.0	99.0	98.1
Trout ♀ × Trout ♂	287	100.0	98.6	96.5
Trout ♀ × Salmon ♂	888	100.0	96.6	96.3*
Salmon ♀ × Trout ♂	489	100.0	0.0	0.0

*These hybrids all died by 30 days after hatching

pattern of the F1 generation was the same as that of trout (Fig. 4H). Furthermore, the F1 generation was fertile to produce normal F2-generation trout (Fig. 4I; Table 5). We thus succeeded in establishing a surrogate broodstock technique for salmonids where spermatogonia are transplanted into sterile triploid xenogeneic recipients to produce a next generation consisting of all donor-derived fish (Okutsu *et al*. 2007). Spermatogonia collected from one donor can be transplanted to >100 recipients. In the present study, spermatogonia were transplanted into 100 recipients. Indeed #1–4 female recipients and all male recipients were prepared using spermatogonia collected from one donor trout. Therefore, juveniles (in total 27) obtained from #1–4 female recipients were produced from a single donor. In other words, these F1 fish were self-fertilized fish produced from one donor trout. This suggests that even when only one male remains, more fish containing both male and female can be produced using the present technique. Furthermore, after cryopreserving fish spermatogonia, a species can be efficiently revived by transplanting cryopreserved spermatogonia into sterile recipients using the present technique. Even if a species has gone extinct, the species can be revived using cryopreserved spermatogonia if the natural habitat is first restored, individuals of the species are produced using the present technique, and those individuals are released to its natural habitat.

In the present study, great individual differences were seen in the development rate of eggs obtained from female triploid salmon

recipients, and rates were low in some recipients (Table 4). Female triploid salmon produce small ovaries (Fig. 4A), and individual differences were attributed to low hormone secretion from the ovary and subsequent abnormal feedback regulations of the hypothalamus and pituitary. In the future, the efficiency of the present technique will be improved by clarifying the secretion profiles of reproductive hormones in female triploid salmon recipients and administering the necessary exogenous hormones.

We propose application of the present results to marine fish, to markedly improve the efficiency of seed production for tuna to counter the rapidly decreasing numbers seen in recent years. Bluefin tuna takes 3–5 years to mature, and since adult bluefin tuna weigh about 100–600 kg, farming requires great facilities, manpower and costs. Mackerel mature in 1–2 years and the small weight of about 500 g allows the fish to be raised in a small tank. If mackerel could be used as a surrogate for bluefin tuna, large quantities of seeds could be produced in small land-based fish tanks over a short period of time. In this manner, the present technique could be used to not only conserve endangered species, but also drastically improve the supply efficiency of marine products that have been in great demand worldwide in recent years.

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