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Production of live fish derived from frozen germ cells via germ cell transplantation

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ABSTRACT

The conservation of endangered fish is an urgent issue. Although cryo-banking of fish gametes might ultimately help conserve endangered fish, cryopreservation of fish eggs or embryos is still not possible due to their large size and high yolk content. Therefore, as an alternative, we focused on undifferentiated germ cells, such as primordial germ cells, spermatogonia, and oogonia, as materials for cryopreservation. Transplantation of cryopreserved germ cells into the body cavity of allogeneic or xenogeneic recipients sterilized by triploidization or endogenous germ cell ablation resulted in migration of the transplanted germ cells toward the recipient genital ridges, where they were eventually incorporated. The donor-derived germ cells initiated either spermatogenesis or oogenesis in the recipient gonads, depending on the sex of the recipient. Furthermore, by mating the male and female recipients, viable offspring derived from the frozen germ cells were produced. Although this technology was established using salmonid fish, we found that it is applicable to a wide range of fish species. Thus, this method might represent a "silver bullet" for preserving the valuable genetic resources of endangered fish species.

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1. Introduction

A number of fish species now face extinction due to overfishing and habitat destruction. Although considerable efforts have been expended for preservation, such as the establishment of closed areas and seasons for fishing, improvements in fish resources have been rather limited. Furthermore, once a fish habitat is destroyed, it is extremely difficult to repair it in a short period of time. This is a particularly serious problem in many river systems. The construction of riparian structures such as dams and the paving of riverbanks with concrete makes it nearly impossible to later return a river ecosystem to its original wild status. Therefore, emergency measure are needed to preserve the valuable genetic resources of endangered fish species. Cryopreservation of fish sperm has become a routine process; however, egg cryopreservation remains very difficult due to the large size and high lipid and yolk content of fish eggs (Mazur et al., 2008). Obviously, sperm alone cannot produce live fish individuals. Although a few reports have described the possibility of cryopreserving fish eggs (Zhang et al., 1989; Chen and Tian, 2005), the reproducibility of the techniques has not been confirmed (Edashige et al., 2006).

As an alternative to fish egg cryopreservation, we focused on the preservation of primordial germ cells (PGCs), which are small enough (most fish eggs are >1 mm in diameter, but PGCs are about 15 μm in diameter) to be cryopreserved and do not contain much lipid and yolk material (Patino and Takashima, 1995). Therefore, we expected that PGCs could be kept in liquid nitrogen semi-permanently, such that whenever fish of a given species are needed, the frozen PGCs could be converted into functional gametes by transplanting them into recipient fish of a closely related species to the donor. Once the recipient fish mature, they would be able to produce donor-derived gametes. Therefore, simply by mating male and female recipients, an endangered or even extinct species could be regenerated solely from frozen genetic material (Fig. 1). The question then became: how do we transplant PGCs into the recipient fish?

2. Intraperitoneal transplantation of primordial germ cells into newly hatched larvae

In order to bring the above-mentioned scenario into reality, allogeneic or xenogeneic transplantation of donor PGCs is necessary, as isogeneic recipients of endangered species are not available. One potential obstacle could be rejection of the donor-derived PGCs by the immune system of the recipient fish. To overcome this potential obstacle, newly hatched larvae were used as recipients, because immediately after hatching, larvae do not have a mature immune system (Manning and Nakanishi, 1996). In other words, we expected that these newly hatched larvae would not have the ability to reject donor-derived foreign cells. In order to perform PGC transplantation into newly hatched larvae, we chose rainbow trout (*Oncorhynchus mykiss*) carrying the

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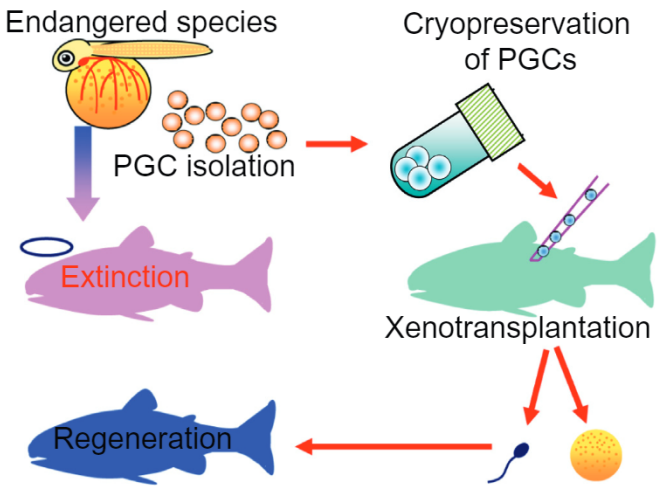

Fig. 1. Principle of egg and sperm production from different species in surrogate parents using germ cell transplantation.

Gfp gene driven by *cis*-elements of the rainbow trout *vasa* gene as the donor (Yoshizaki et al., 2000; Takeuchi et al., 2002) and wild type rainbow trout as recipients. The salmonid species were chosen because the total length of newly hatched larvae carrying sexually undifferentiated PGCs is approximately 1.5 cm, which is much larger than other conventional model fish species, such as zebrafish and medaka, the newly hatched larvae of which are about 5 mm in length and thus extremely difficult to dissect surgically.

Donor PGCs were harvested from hatching stage (32 days post-fertilization, dpf) larvae. In rainbow trout, PGC migration is almost completed by around 20 dpf (Sawatari et al., 2007), and at 30 dpf, almost all PGCs are incorporated into the genital ridges, making it easy to harvest PGCs simply by isolating the genital ridges surgically (Takeuchi et al., 2003). After enzymatic dissociation, approximately 3000 genital ridge cells containing 10–20 PGCs was transplanted into the newly hatched recipient larvae. Initially, we tried to transplant the PGCs into the recipient genital ridges; however, they were too small for microinjection. In all vertebrates, PGCs emerge from the extra-gonadal area and then migrate toward the genital ridges, where they eventually coalesce with gonadal somatic cells (Richardson and Lehmann, 2010). Because this migration is at least partly regulated by chemotaxis (Richardson and Lehmann, 2010), we expected that if donor-derived PGCs were transplanted into the recipient body cavity (Fig. 2), the PGCs would migrate toward the recipient gonadal anlagen. Indeed, we found that intraperitoneally transplanted PGCs migrate toward and are eventually incorporated into the recipient genital ridges in approximately 20% of the recipient larvae receiving transplants (Takeuchi et al., 2003). The transplanted PGCs then begin proliferation and gametogenesis in the recipient genital ridges, and 2 to 3 years later, the recipient wild type rainbow trout produce donor-derived eggs and sperm, depending on the sex of the recipient fish (Takeuchi et al., 2003). This sexual bi-potency of the fish germ cells can be explained by the fact that the X and Y chromosomes in fish are highly similar, except at the male-determining locus (Yano et al., 2012).

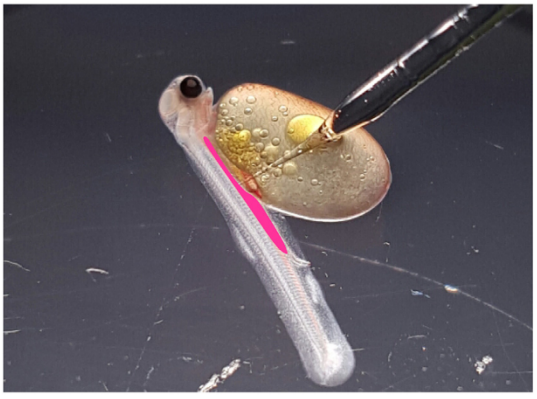

Fig. 2. Microinjection of germ cells into the peritoneal cavity of newly hatched trout embryos. The location of the peritoneal cavity is shown in pink. A pair of genital ridges (approximately, 3.5 mm in length and 60 μm in diameter) are located on the dorsal wall of the peritoneal cavity. Total length of the recipient embryos are approximately 15 mm.

In order to confirm the functionality of the gametes produced by the recipient rainbow trout, progeny tests were performed using gametes from both recipients and wild type rainbow trout. In this study, dominant albino *vasa-Gfp* transgenic and wild type non-transgenic fish were used as donors and recipients, respectively. As a result, among the wild type F1 larvae, both albino and GFP-positive larvae were obtained. The germ line transmission rate of the donor-derived haplotype was 2–4%. During the experiment, we also found that PGCs harvested from genital ridges migrated when transplanted into the body cavity of the recipient hatchlings but not when transplanted into the blastulae. On the other hand, Saito et al. (2010) reported that migrating PGCs harvested from somite-stage embryos migrate when transplanted into zebrafish blastula embryos. Thus, ability of PGCs to migrate changes dramatically during their development.

We next performed xenogeneic transplantation of rainbow trout PGCs into newly hatched larvae of masu salmon (*O. masou*) (Takeuchi et al., 2004). The rainbow trout and the masu salmon belong to the same genus, *Oncorhynchus*, and their estimated genetic distance is approximately 8 million years (McKay et al., 1996). Intraperitoneally transplanted rainbow trout PGCs were successfully incorporated into masu salmon gonads, and the masu salmon recipients produced functional sperm derived from the donor rainbow trout. By in vitro fertilization of eggs obtained from wild type rainbow trout and milt obtained from the recipient masu salmon, offspring were produced. As the hybrids produced by rainbow trout eggs and masu salmon sperm were not viable, only pure rainbow trout produced by donor-derived rainbow trout sperm were expected to survive. As a result, most of the F1 offspring (99.6%) became non-viable hybrids and did not complete yolk absorption; however, a total of 0.4% of the F1 offspring swam-up and developed normally. Further, the all survived hatchlings carried the *vasa-Gfp* gene, which is a genetic marker of the donor fish. The donor-derived rainbow trout showed normal external morphology and matured normally. In addition, DNA fingerprint analyses reveled that donor-derived rainbow trout showed an identical RFLP pattern to wild type rainbow trout but clearly distinctive from that of masu salmon. Thus, we confirmed that the PGC transplantation technology developed by our group is suitable for xenotransplantation.

3. Intraperitoneal transplantation of spermatogonia harvested from adults

In order to apply the PGC transplantation technology described above to a real conservation project, we needed to collect large numbers of larvae carrying PGCs. In general, finding such a small-sized fish is not easy, especially around the spawning grounds of salmonids, such as mountain streams. Furthermore, most of the newly hatched larvae of marine fishes or non-salmonid freshwater fishes are about 3–5 mm in total length, and species identification is also very difficult. Therefore, we needed to find another alternative donor cell type instead of PGCs for practical applications. As a candidate cell type, we focused on type A spermatogonia (ASG) (Okutsu et al., 2006). Adult *vasa-Gfp* rainbow trout was used as the donor for spermatogonial transplantation in this study. Approximately 18,000 testicular cells containing 10,000 ASGs were microinjected into the body cavity of newly hatched wild type rainbow trout larvae. Eighteen days after transplantation, donor-derived ASGs were incorporated into the recipient genital ridges in

approximately 40% of the recipient fish that received transplants, suggesting that the ASGs isolated from adult testes retain the ability to migrate toward the recipient gonadal anlagen in the larval body cavity. The mean number of ASGs incorporated per larva was about five. Although it was difficult to perform direct comparisons, nearly 1000 times more ASGs were transplanted into the recipient body cavity compared with the number of PGCs used for transplantation (Takeuchi et al., 2003), with a doubled success rate (40% with ASGs and 20% with PGCs). Thus, although the migration ability of ASGs might be much weaker than that of PGCs, at least some retain the ability to migrate toward the recipient gonads. Indeed, we often found ASGs extending pseudopodia near the genital ridges of the recipient fish (Yoshizaki et al., 2012).

Two to three years after transplantation, nearly 50% of the recipient males were confirmed to produce donor-derived sperm, as determined by PCR analyses using specific primers against the *Gfp* gene. Furthermore, progeny tests confirmed that the germ line transmission rate of the donor-derived haplotype was about 5.5%. The donor-derived offspring showed normal early survival rates and grew up normally, at least as determined based on observations of external morphology (Okutsu et al., 2006).

To our surprise, the ASGs transplanted into female larvae also migrated toward the recipient ovarian anlagen and initiated proliferation. Six months after transplantation, the donor-derived green germ cells had differentiated into oocytes with a diameter of approximately 100 μm (Okutsu et al., 2006). Two to three years after transplantation, progeny tests were performed using recipient females that received ASGs from dominant albino transgenic (*vasa-Gfp*) and wild type non-transgenic males. The resulting offspring produced by approximately 40% of the female recipients contained larvae showing albino and GFP-positive phenotypes. The germ line transmission rate of the donor-derived haplotype was approximately 2%. These offspring produced using eggs derived from donor ASGs developed and matured normally. Indeed, the hatching and swimming-up rates did not significantly differ compared with control fish.

Previously, it was thought that the sex of young larvae could be easily reversed before completion of sexual differentiation by application of exogenous sex steroids (for both feminization and masculinization) or aromatase inhibitors (for masculinization), since their sex chromosomes have not diverged much. However, it is now widely held that the sex of the fish cannot be manipulated after the morphological sex differentiation period (Devlin and Nagahama, 2002). This study clearly proved that at least the germ cells (probably germ line stem cells) demonstrate a high level of sexual plasticity and that the sex of the germ cells is determined primarily by the somatic environment rather than in a cell-autonomous manner (Fig. 3) (Okutsu et al., 2006).

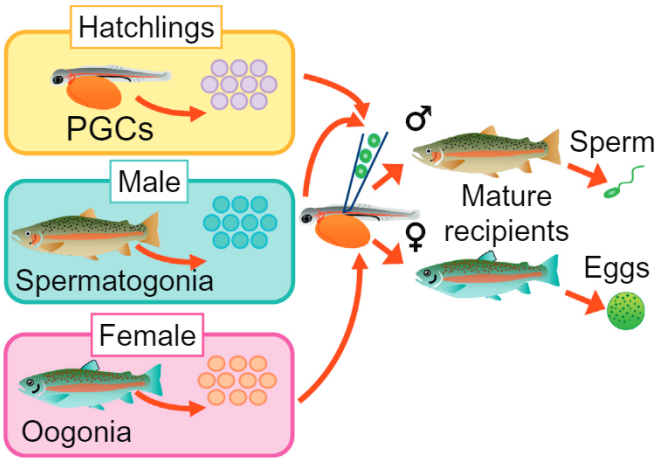

Fig. 3. Production of donor-derived gametes using recipients receiving primordial germ cells, spermatogonia, or oogonia. Regardless of the genetic sex of the donor germ cells, the recipients produced donor-derived eggs or sperm, depending on the sex of the recipient.

Another important biological finding resulting from this experiment pertained to the stemness of the donor-derived ASGs. Among the recipients that received transplants, 42.9% showed incorporation of donor ASGs into recipient gonads, and 45.6% showed proliferation of the donor ASGs. Furthermore, 43.9% of recipients that received transplants produced functional gametes (either eggs or sperm) during the spawning season. Most of the recipient fish repeatedly produced donor-derived gametes, for at least two consecutive spawning seasons (Okutsu et al., 2006) (in the different experiments, we also confirmed production of donor-derived gametes in three consecutive spawning seasons). Notably, male recipients produced >200 million sperm every year, suggesting that these donor-derived ASGs behave as spermatogonial stem cells (SSCs). In addition, the fact that the percentages of recipient fish producing donor-derived gametes and those showing donor germ cell incorporation and proliferation were quite similar suggests that most or even all of the ASGs that are incorporated into the recipient gonads can behave as SSCs in the recipient gonads. This finding indicates that germ cell transplantation is a powerful tool for use in the functional characterization of SSCs in fish (Okutsu et al., 2006), as previously reported in rodents (Brinster, 2002).

4. Intraperitoneal transplantation of oogonia harvested from adults

As the next step, we performed oogonial transplantation. It is widely known that the ovaries of adult female fish retain large numbers of mitotic germ cells and that at least some of these cells can behave as oogonial stem cells (Nakamura et al., 2010). The protocol used for rainbow trout ASG transplantation was therefore applied to oogonial transplantation (Yoshizaki et al., 2010). The intraperitoneally transplanted oogonia migrated toward the recipient gonads, where they were eventually incorporated. The donor-derived oogonia initiated spermatogenesis and oogenesis in male and female recipients, respectively, and eventually produced functional gametes (Fig. 3). Thus, oogonia are capable of differentiating into functional sperm, suggesting that oogonia also exhibit sexual plasticity. Furthermore, successful intraperitoneal transplantation of oogonia has been demonstrated in zebrafish, with the resulting male recipients producing sperm derived from the transplanted oogonia (Wong et al., 2011).

In this study, all F1 offspring produced by male recipients were females, suggesting that the XX oogonia differentiate into functional X-sperm and that the Y chromosome is not necessary for whole spermatogenesis and spermiogenesis, at least in rainbow trout (Yoshizaki et al., 2010). In the above-mentioned ASG transplantation studies, we also found that XY ASG can produce functional eggs with either X or Y chromosomes (Okutsu et al., 2015). The Y eggs produced by the female recipients could be fertilized with Y sperm to produce YY males, which in turn produced only Y sperm; consequently, the next generation were all males carrying an XY chromosome set. These results support the proposed hypothesis that aside from the male-determining locus, X and Y chromosomes are at least functionally equivalent (Yano et al., 2012, 2013).

A recent report described sex-dependent dimorphism of gene expression profiles in medaka mitotic germ cells (Nishimura et al., 2015), but our results suggest that these sex-specific gene expression profiles can be overwritten by the somatic environment created by recipient gonads. Because we have not performed any molecular-level analyses of donor germ cell gene expression profiles, we need further precise analyses in order to elucidate the molecular mechanism of sex reversal in immature mitotic germ cells.

Another interesting result of the oogonial transplantation study was that the oogonia incorporated into the recipient genital ridges did not change their morphological characteristics to match those of PGCs (Yoshizaki et al., 2010). Although oogonia and ASGs exhibit a very similar morphology (diameter of both cells is 8–10 μm), the morphology of

PGCs is clearly distinctive (diameter of 15–20 μm). When we analyzed the morphological changes in donor-derived oogonia incorporated into the recipient genital ridges, their morphology was always similar to that of the donor oogonia, and they were consistently smaller than endogenous PGCs located adjacent to the donor-derived oogonia (Fig. 4). Based on these data, we speculate that the donor-derived oogonia initiate spermatogenesis in the male recipients without de-differentiation into sexually undifferentiated PGCs.

5. Transplantation of germ cells into sterile recipients

As described above, we developed PGC, ASG, and oogonium transplantation techniques using salmonid species as a model; however, the fish that received donor-derived germ cells produced a considerable number of their own recipient gametes together with donor-derived gametes. Therefore, we developed another method to produce surrogate recipients capable of producing only donor-derived gametes. As sterile recipients incapable of producing their own gametes, we chose triploid fish. In lower vertebrates, including fishes, triploids can be produced quite easily by suppressing the extrusion of the second polar body using temperature shock or pressure shock (Donaldson, 1986). The resulting embryos carrying two sets of maternal chromosomes and one set of paternal chromosomes develop normally but become sterile due to abnormal disjunction of chromosomes during meiosis. This triploidization has been widely used in aquaculture because gonadal growth often suppresses somatic growth, which is important for obtaining the maximum amount of filet. Therefore, ASGs of *vasa-Gfp* rainbow trout were transplanted into triploid masu salmon larvae (Okutsu et al., 2007). Although triploid male salmonid fishes often produce aneuploid sperm that lack developmental ability, the females are completely sterile. By contrast, the triploid masu salmon that received transplants matured and produced large numbers of haploid sperm and eggs. By in vitro fertilization of the resulting eggs and sperm, we were able to obtain rainbow trout offspring.

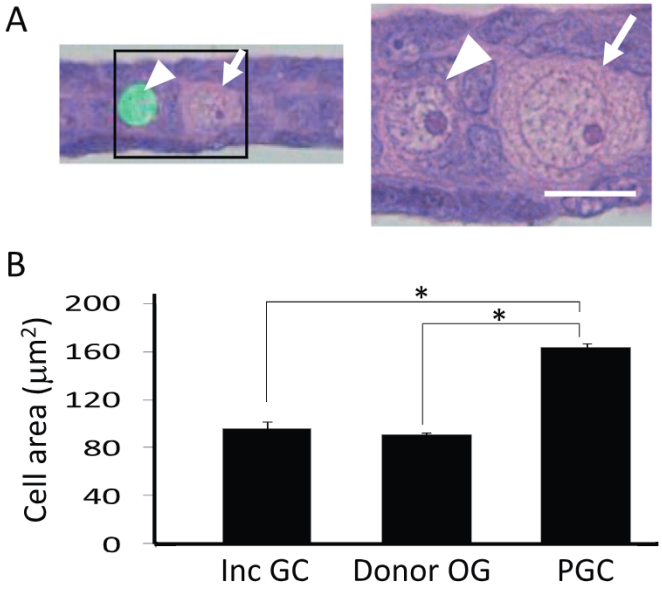

Fig. 4. Donor-derived oogonia directly start gametogenesis without de-differentiating into primordial germ cells in the recipient genital ridges. A) A cross-section of donor oogonia that were incorporated into the recipient genital ridge. The arrowheads indicate donor-derived germ cells stained with GFP antibody, and arrows indicate the endogenous PGCs of the recipient fish. The boxed area is shown at high magnification on the right. B) The cross-sectional areas of donor-derived oogonia that were incorporated into the recipient genital ridges, those of oogonia of 9-month-old donor fish, and those of PGCs of hatching-stage embryo were measured and compared. *, $P < 0.01$. Data are the mean ± s.e.m. ($n = 12$). Scale bar: 10 mm (Yoshizaki et al., 2010).

Masu salmon and rainbow trout require approximately 40 and 32 days, respectively, to reach the hatching stage. All of the resulting offspring produced by masu salmon recipient parents hatched out at exactly the same time as the rainbow trout (32 days). Furthermore, the resulting offspring carried the *vasa-Gfp* gene and exhibited identical DNA fingerprints as the donor rainbow trout but were clearly distinctive from the masu salmon with respect to these characteristics. More importantly, the external morphology of the resulting F1 juveniles was identical to that of control rainbow trout. Therefore, we concluded that triploid masu salmon recipients produce only donor-derived rainbow trout offspring. This observation indicates that triploid fish have a normal ability to nurse diploid germ cells; once the diploid germ cells are incorporated into the recipient genital ridges, gonadal somatic cells and their endocrine system functions normally until the donor-derived germ cells mature and produce gametes.

Although we found that these triploid masu salmon showed quite high fecundity (number of sperm and eggs produced by fully mature recipients was equivalent to the number of gametes produced by control diploid masu salmon) (Yoshizaki et al., 2011), it is possible that there was competition between the transplanted germ cells and the recipient triploid germ cells in the recipient gonads, as triploid gonads have a normal number of mitotic germ cells even though they cannot proceed to meiosis normally and mature. Therefore, we examined recipients lacking germ cells produced by knock down of the *dead end* (*dnd*) gene using antisense morpholino oligonucleotides (Yoshizaki et al., 2016). This methodology was first developed in zebrafish, and the resulting germ cell–free recipients were utilized in primordial germ cell transplantation (Saito et al., 2008). In experiments with rainbow trout, most of the resulting embryos lacked germ cells simply as a result of microinjecting fertilized eggs with antisense morpholino oligonucleotide against the *dnd* gene. Although germ cell–free zebrafish and medaka all become males (Weidinger et al., 2003; Slanchev et al., 2005; Kurokawa et al., 2007; Tzung et al., 2015), the sex ratio of the germ cell–free rainbow trout was 50:50, which is a favorable characteristic in a recipient used for germ cell transplantation.

Germ cell transplantation studies using the germ cell–free recipients revealed that the transplanted ASGs colonized the recipient gonads more effectively compared with control recipients carrying endogenous germ cells (Yoshizaki et al., 2016), suggesting that the recipient genital ridges harbor a germ cell niche; by removing the endogenous germ cells, the available niche for the donor-derived germ cells would thus expand. Furthermore, the donor-derived germ cells incorporated into the recipient gonads exhibited a significantly higher proliferation rate compared with that of donor-derived germ cells transplanted into control recipients carrying endogenous germ cells (Yoshizaki et al., 2016). The germ cell–free recipient masu salmon that received rainbow trout germ cells eventually produced a similar number of rainbow trout eggs and sperm as control masu salmon (Yoshizaki et al., 2016). Although we could not perform a direct comparison of the efficiency of donor-derived gamete production between the germ cell–free and triploid recipients, germ cell–free recipients could be a powerful tool for use in species in which the triploids can produce a non-negligible number of aneuploidy gametes, such as grass puffer (Hamasaki et al., 2013, 2017).

Genome editing by clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 can be employed in a variety of fish species. A recent report described the production of germ cell–free Atlantic salmon (*Salmo salar*) using genome editing (Wargelius et al., 2016). The genome editing method could become a powerful tool for producing germ cell–free recipients (Li et al., 2017) in the future. Another recent report described the treatment of fertilized eggs with a membrane-permeable morpholino oligomer against *dnd* for the mass production of germ cell–free fish using zebrafish (Wong and Zohar, 2015). With the genome editing method, only one-fourth of the offspring produced by mating heterozygous *dnd* mutant males and females become homozygous *dnd* mutants lacking germ cells. In addition, producing germ cell–free fish by microinjecting RNAs (for direct

knock out in the founder generation) or morpholino antisense oligonucleotides into each fertilized eggs is both labor-intensive and time-consuming and thus impractical. As such, mass production of sterile fish using membrane-permeable morpholino oligomers is an attractive alternative for creating germ cell–free recipients for use in germ cell transplantation in fish.

6. Transplantation of cryopreserved germ cells

Thus, we established germ cell transplantation methods using various donor germ cells. We then performed transplantation of cryopreserved germ cells. The initial step was to optimize the cryopreservation protocol. Limited availability of requisite equipment and facilities represents a potential problem for the cryopreservation of germ cells isolated from endangered or economically valuable species. Therefore, we decided to cryopreserve whole testes rather than dissociated testicular cells or purified ASGs, which require enzymatic treatment and several centrifugation steps in preparation for freezing. Using this approach, we successfully cryopreserved whole testes (testis weight, 0.014 ± 0.001 g) of juvenile rainbow trout using a cryomedium composed of dimethyl sulfoxide, trehalose, and chicken egg yolk (Lee et al., 2013). The testes were equilibrated in the cryomedium on ice for 1 h, after which they were slowly frozen (−1 °C/min) in a cryo-container (e.g., BiCell, Nihon-Freezer, Tokyo, Japan) kept in a deep-freezer set at −80 °C. After 90 min of slow freezing, the samples could be stored in liquid nitrogen at least for 3 years without any decline in the cell survival rate. In independent studies, we also found that whole testes can be stored in liquid nitrogen for 5 years without any decline in the ASG survival rate (Lee et al., 2016a). Cryopreserved ASGs can be used for intraperitoneal transplantation using the methods described above. The transplanted ASGs are capable of migrating toward recipient gonads, into which they are subsequently incorporated. The donor-derived ASGs initiate proliferation and spermatogenesis in male trout recipients and oogenesis in female trout recipients (Table 1) (Lee et al., 2013). These spermatogonial transplantation techniques can also be used in masu salmon recipients (Lee and Yoshizaki, unpublished data).

In real-world conservation projects, we often face a significantly biased sex ratio in donor fish. In these cases, cryopreservation of testes and subsequent spermatogonial transplantation is not always adequate. As such, we also examined methods for ovarian cryopreservation and transplantation using rainbow trout (Lee et al., 2016b). Again, we used whole ovaries rather than dissociated ovarian cells for cryopreservation. The frozen ovaries could be stored in liquid nitrogen for at least for 1185 days without any decline in the oogonial survival rate. Furthermore, the oogonia isolated from the cryopreserved ovaries can be used for transplantation into both allogeneic (Table 1) and xenogeneic (masu salmon) recipients. Thus, methods for the cryopreservation of whole testes and ovaries were established in addition to protocols for producing both functional eggs and sperm via germ cell transplantation into allogeneic and xenogeneic recipients. As discussed earlier, fish eggs and embryos cannot be cryopreserved and sperm alone cannot produce live fish individuals. Since spermatogonia and oogonia possess high level of sexual plasticity and have abilities to produce both eggs and sperm, cryopreservation of these mitotic germ cells can be a suitable alternative to the cryopreservation of fish eggs or embryos.

It is notable that one testis isolated from a 20- to 30-g juvenile rainbow trout can supply millions of ASGs, which is sufficient for transplantation into dozens of recipients. As typically almost half of recipient fish receiving donor-derived germ cells can produce donor-derived eggs or sperm (Table 1), this technology could become a powerful tool for use in future efforts to regenerate extinct fish species.

We also recently reported the freezing of “whole fish” in a deep freezer (Lee et al., 2015). There are two prerequisites for successful cryopreservation of cells: 1) the use of appropriate cryoprotectants, and 2) selection of an appropriate cooling rate. Regarding the cooling rate, there are two options: rapid cooling used for vitrification and slow freezing. In the case of freezing whole fish in a deep freezer, vitrification is impossible, as the mass of the fish body is too large to allow for vitrification of the germ cells, which are located almost in the middle of the body, which leads to a slower cooling rate, even though the whole fish is immersed directly in liquid nitrogen. Indeed, no viable germ cells were retrieved from the gonads when whole fish were immersed in liquid nitrogen (Lee et al., 2015). Therefore, we simply stored whole rainbow trout in a − 80 °C deep freezer. Using this approach, we were able to mimic the slow freezing of testes, with the testes cooling at an ideal rate of approximately −1 °C/min. Most likely, the body of the fish functions as a thermal insulation material for the testes. Viable ASGs could be retrieved from the testes of the frozen fish after thawing. The ASGs were still transplantable and differentiated into functional sperm or eggs, depending on the sex of the recipient fish. By mating the recipient triploid masu salmon parents, we obtained viable rainbow trout offspring derived from the frozen donor fish (Lee et al., 2015). It is noteworthy that the survival rate of the ASGs did not decline after 3 years in a deep freezer. We also found that trout serum exhibits a cryoprotectant activity, and this was also an important factor in our ability to retrieve viable ASGs from the frozen fish. However, the survival rate of the ASGs retrieved from frozen whole fish (Lee et al., 2015) was much lower than that of ASGs retrieved from testes frozen with appropriate cryoprotectants (Table 1) (Lee et al., 2013). Therefore, if a well-equipped laboratory is available for germ cell cryopreservation, whole-gonad cryopreservation should be the first option. On the other hand, if cryopreservation must be carried out in a hatchery or fish farm lacking the appropriate equipment, whole-body freezing might be the best option (Fig. 5). In the case of an emergency situation, such as the sudden die off of economically valuable or endangered fish in a fish-rearing facility due to disease or accident, the whole fish can be stored in a deep freezer until the specimens can be sent to a laboratory that routinely performs spermatogonial transplantation.

Table 1
Comparison of various cryopreservation methods for rainbow trout germ cells.

Donor cells	Survival of germ cells after cryopreservation (%)	No. of ASGs used for transplantation	% of recipient fish producing donor-derived gametes[h]
Cryopreserved testis[a]	35.1[d]	5000	♂ 47.1 ♀ 42.9
Control testis[a]	–	5000	♂ 45.8 ♀ 38.9
Frozen fish[b]	0.14[e]	500[f]	♂ 21.7 ♀ 25.0
Control testis[b]	–	500[f]	♂ 36.8 ♀ 28.6
Cryopreserved ovary[c]	72.9[d]	10,000[g]	♂28.0 ♀20.0
Control ocvary[c]	–	10,000[g]	♂30.8 ♀18.2

[a] Lee et al., 2013
[b] Lee et al., 2015
[c] Lee et al., 2016b.
[d] Since the majority of cryoinjured cells were lysed by protease activity during the dissociation procedure and the total numbers of ASGs within the right and left testes of a given individual were almost identical, survivals of germ cells after cryopreservation were obtained by the following formula: Survival (%) = No. of GFP-positive germ cells retrieved from a cryopreserved left gonad/No. of GFP-positive germ cells retrieved from a non-frozen right gonad.
[e] Predicted value obtained by the following formula: Survival (%) = No. of GFP-positive germ cells retrieved from a cryopreserved whole fish/2,000,000 (predicted number of ASGs retrieved from a non-frozen fish).
[f] Small number of germ cells were transplanted since their survival was quite low after freezing and thawing of whole fish.
[g] Larger number of germ cells were transplanted since it was revealed that transplantation efficiencies of oogonia was lower than those of spermatogonia (Yoshizaki et al., 2010).
[h] These are the results using 2.5–3-year-old triploid allogeneic recipients.

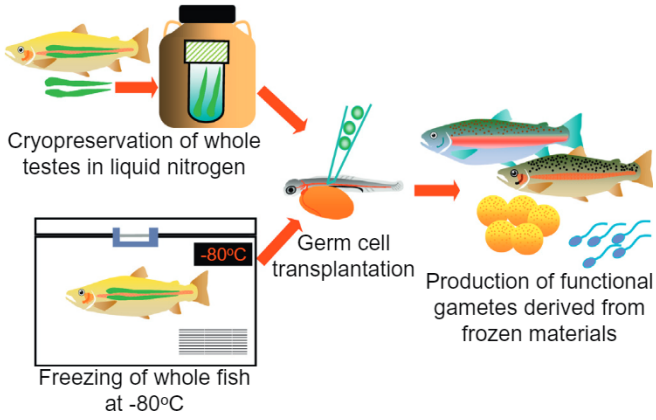

Fig. 5. Application of spermatogonial transplantation to the conservation of endangered fish species. Restoration of an extinct species could be achieved by transplanting spermatogonia retrieved from cryopreserved whole testes or whole fish into recipient hatchlings of a closely related species.

Some researchers have suggested that as long as somatic cells are cryopreserved, live animals (including fish) can be regenerated using nuclear transplantation technology (Mastromonaco et al., 2014). However, only nucleus-cytoplasm hybrids carrying mitochondrial DNA derived from the recipient species can be produced, and these animals are not suitable for return into wild environments. By contrast, the method described in this review enables the production of complete fish in which the nuclear and mitochondrial DNA are derived from the donor fish species. To date, we have begun several conservation projects involving several endangered salmonid species by creating a cryo-bank of whole testes.

7. Application of the germ cell transplantation system to other species

The principal biological mechanisms underlying the above-mentioned intraperitoneal germ cell transplantation method (i.e., newly hatched embryos do not possess a mature immune system, and germ cells can seek out the recipient gonads and migrate to them) have been conserved throughout vertebrate evolution. Therefore, the intraperitoneal germ cell transplantation method using newly hatched embryos as recipients can be applied to a wide range of fish species. Indeed, we successfully applied the technique to nibe croaker (*Nibea mitsukurii*) (Takeuchi et al., 2009; Higuchi et al., 2011; Yoshikawa et al., 2017), a species belonging to the Scianidae family; chub mackerel (*Scomber japonicus*) (Yazawa et al., 2010), belonging to the Scombridae family; yellowtail (*Seriola quiqueradiata*) (Morita et al., 2012) and horse mackerel (*Trachurus japonicus*) (Morita et al., 2015), belonging to the Carangidae family; tilapia (*Oreochromis niloticus*) (Farlora et al., 2014), belonging to the Cichlidae family; medaka (*Oryzias latipes*) (Seki et al., 2016), belonging to the Adrianichthyidae family; and tiger puffer (*Takifugu rubripes*) (Hamasaki et al., 2017), belonging to the Tetraodontidae family. In all cases, intraperitoneally transplanted ASGs showed no signs of immune rejection and migrated to the recipient genital ridges. Nibe croaker (Yoshikawa et al., 2017), yellowtail (Morita et al., 2012), tilapia (Farlora et al., 2014), and medaka (Seki et al., 2016) recipients that received allogeneic donor germ cells completed donor-derived gametogenesis. Insemination experiments confirmed that the resulting gametes were all functional and produced normal offspring. In the case of nibe croaker, efficient production of only donor-derived eggs and sperm from triploid surrogate females and males, respectively, was confirmed (Yoshikawa et al., 2017). Not only was the proportion of donor-derived offspring in the F1 generation improved using the triploid recipients, the frequency of recipients that produced donor-derived gametes increased about 7-fold for male recipients and 4-fold for female recipients when triploids were used as recipients instead of diploids. Thus, we confirmed that the triploid recipient is a powerful tool for efficient production of surrogate broodstock, not only in salmonids, but also in scianids. By applying the method established in salmonids to cryopreserved germ cells of these various fish species, long-term preservation of genetic resources could become possible in the near future.

Recently, we also found that horse mackerel recipients that received yellowtail testicular cells (intra-family and inter-genus transplantation) produced donor-derived functional sperm (Morita et al., 2015). Further, grass puffer (*Takifugu niphobles*) recipients that received tiger puffer germ cells produced both donor-derived xenogeneic eggs and sperm (Hamasaki et al., 2017). Thus, we confirmed that xenogeneic germ cell transplantation is possible not only in salmonids, but also in carangids and tetraodontids, which include various commercially important species. In recent years, resources of Atlantic bluefin tuna (*Thunnus thynnus*) and Southern bluefin tuna (*T. maccoyii*) have declined to the point that these species have been added to the Red List. One solution to reverse this decline is marine ranching using artificial seed. However, the bluefin tunas take 3 to 10 years to mature, and since broodstock individuals often weigh >100 kg (market size is much smaller), seed production of bluefin tuna requires significant resources in terms of facilities, manpower, and money. On the other hand, mackerel (*Scomber japonicus*) mature in 1 year, and their low individual weight of about 300 g allows the fish to be raised in small, land-based tanks. If mackerel could be used as a surrogate for bluefin tuna, large quantities of seed could be produced in such land-based fish tanks in a short period of time. This technology is expected to accelerate the breeding process of these commercially important fish species. Currently, we are developing the basic technology to establish a surrogate broodstock system for the production of bluefin tuna.

8. Perspectives

In this review, we described a germ cell transplantation technology established using newly hatched larvae as recipients. Successful transplantation of spermatogonia into the testes of adult recipients was recently performed in tilapia (Lacerda et al., 2010), pejerrey (Majhi et al., 2009), and zebrafish (Nóbrega et al., 2010). Although the reason why donor-derived germ cells are not immuno-rejected by the allogeneic or xenogeneic recipients is unknown, these germ-cell transplantation systems could also be useful in the creation of surrogate broodstock and preservation of genetic resources in fish. As mentioned above, transplantation of PGCs is now possible in several fish species. In addition to intraperitoneal transplantation of gonadal PGCs into newly hatched larvae, transplantation of migration-stage PGCs into the blastodisc of blastula embryos is also now possible (Saito et al., 2008; Yasui et al., 2011; Goto et al., 2012; Li et al., 2016). In cases in which donor embryos with migrating PGCs are available, the technology described here could be applied to the conservation of endangered fish species. It is noteworthy that techniques for the cryopreservation of whole embryos carrying migrating PGCs are already established for cyprinid species (Higaki et al., 2010; Inoue et al., 2012).

Furthermore, the development of methods for the in vitro culture of spermatogonia in several fish species could improve the efficiency of germ cell transplantation, as donor cells could be amplified in vitro prior to use (Hong et al., 2004; Shikina et al., 2008; Shikina and Yoshizaki, 2010; Lacerda et al., 2013). For some species of small fish that cannot supply an adequate number of donor germ cells for transplantation, in vitro propagation of donor germ cells represents a potential solution. Furthermore, we recently discovered that germ cells cultured for short periods of time provide better transplantation results than freshly prepared germ cells (Shikina et al., 2013). Freshly prepared germ cells could be damaged by proteinases during the testis dissociation procedure, and short-term culture prior to transplantation could enable healing of this damage. Other recent improvements regarding

germ cell manipulation include the identification of a cell-surface protein that is predominantly expressed in rainbow trout and bluefin tuna spermatogonia (Nagasawa et al., 2010, 2012); this protein could be used for enrichment of spermatogonia from fish testes using specific antibody-mediated flow cytometry or magnetic cell sorting. Finally, flow cytometry protocols to enrich ASGs using light-scattering properties (Kise et al., 2012; Ichida et al., 2017) and staining with Hoechst 33,342 (Hayashi et al., 2014) could contribute significantly to both basic and applied germ cell biology. In particular, these germ cell enrichment techniques could be particularly useful in cases in which transgenic strains carrying GFP-labeled germ cells are not available, including the target species for most real-world conservation and aquaculture applications.

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