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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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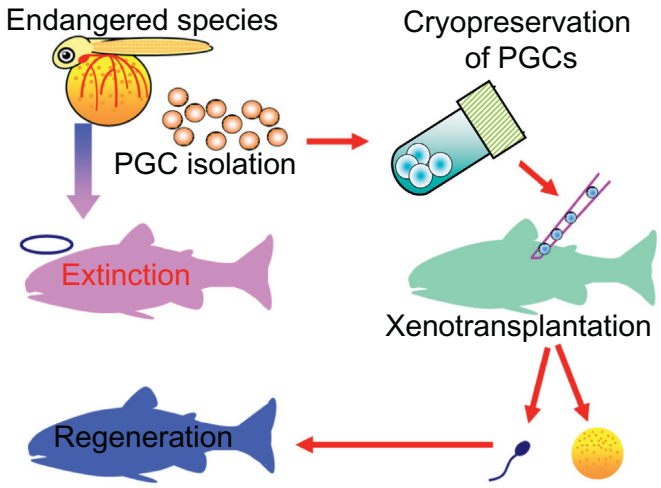

Fig. 1. Principle of egg and sperm production from different species in surrogate parents using germ cell transplantation.

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).

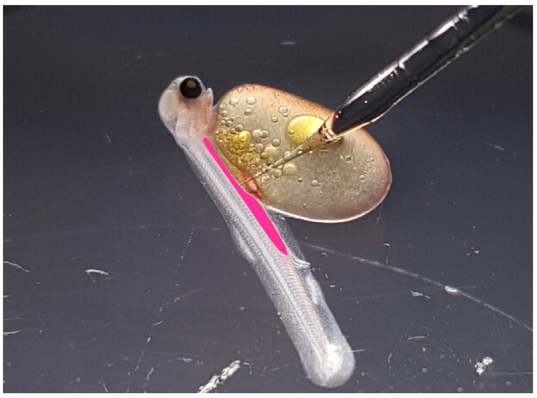

Fig. 2. Microinjection of germ cells into the peritoneal cavity of newly hatched trout embryos. The location of the peritoneal cavity is shown in pink. A pair of genital ridges (approximately, 3.5 mm in length and 60 μm in diameter) are located on the dorsal wall of the peritoneal cavity. Total length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

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