Preservation of fish genetic resources by spermatogonial cryopreservation

［課程博士・論文博士共通］

博士学位論文内容要旨
Abstract

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For the thousands of fish species that are currently threatened with extinction, the rapid development of practical conservation strategies is of crucial importance. The use of captive breeding programs is currently the most widely used method, and it carries with it several risks related to problems in rearing systems. For salmonids in particular, the time required to introduce organisms reared in captivity into their natural habitats is significant. This is the result of associated processes that must be undertaken alongside captive rearing, such as dam decommissioning and improvement of water quality. Therefore, during the resultant prolonged periods of captive breeding, fish can potentially lose genetic diversity and fitness for their natural habitats. Cryobanking allows for the long-term preservation of fish species and their genetic diversity, and could therefore be a valid alternative to, or represent an enhancement of, captive breeding programs. However, past attempts at cryopreservation of fish embryos and mature oocytes have been unsuccessful, as a result of their large size, high yolk content, and low membrane permeability. Although androgenesis performed by using frozen sperm and γ-ray–inactivated xenogenic eggs can regenerate live fish, their survival rate is extremely low, and resulting offspring become nuclear–cytoplasmic hybrids. The loss of maternally inherited materials including mitochondrial DNA makes this method impractical. Previous studies outlined a surrogate broodstock technology used to produce donor-derived eggs and sperm by transplanting germ cells into sterile triploid recipients in salmonids. Intraperitoneally transplanted spermatogonial stem cells (SSC) migrated toward, and were eventually incorporated into, recipient gonads. The transplanted SSC resumed oogenesis and spermatogenesis in female and male recipients, respectively, and ultimately differentiated into functional eggs or sperm. Therefore, the objective of this dissertation was to produce functional eggs and sperm from cryopreserved whole testes or frozen whole fish through intraperitoneal transplantation of frozen testicular cells.

[Chapter 1] The conservation of endangered fish is of critical importance. Cryobanking could provide an effective backup measure for use in conjunction with the conservation of natural populations; however, methodology for cryopreservation of fish eggs and embryos has not yet been developed. The present study established a methodology capable of deriving functional eggs and sperm from frozen type A spermatogonia (ASG). Whole testes taken from hemizygous p*vasa-Gfp* transgenic rainbow trout were slowly frozen in a cryomedium containing 1.3 M DMSO and 0.1 M trehalose, and the viability of ASG within these testes did not decrease over a 728-days freezing period [viability at 1 day (35.1 ± 5.3%) and at 728 days (33.5 ± 7.1%)]. Frozen-thawed SSC that were intraperitoneally transplanted into wild-type triploid hatchlings migrated toward, and were incorporated into, recipient genital ridges. Between 31 and 50 days post-transplantation (pt), the SSC began to proliferate and formed colonies within the recipient gonads. At 182 days pt, GFP-positive oocytes, which were derived from frozen donor ASGs, were found within the recipient ovary. Colonization and proliferation efficiencies of donor-derived ASG within recipient gonads as well as numbers of incorporated ASG were not significantly different among ASG cryopreserved for periods of 1,

24, and 939 days and freshly prepared ASG. Although none of the triploid females that did not undergo transplantation matured, six of 14 female recipients (42.9%) and six of 13 female recipients (46.2%) reached sexual maturity at 3 and 4 years pt, respectively. Although the majority of triploid males that did not undergo transplantation did not produce milt, a small number produced transparent milt containing only aneuploid sperm. Whereas four of 21 male recipients (19.0%), eight of 17 male recipients (47.1%) and nine of 16 male recipients (56.3%) produced white-colored milt at 2, 3 and 4 years pt, respectively. Developmental performances of the gametes obtained from recipients were not significantly different from those obtained from control recipients and wild-type trout. Fertilization of resultant gametes resulted in the successful production of normal, frozen ASG-derived offspring. The methodology of intraperitoneally transplantation of cryopreserved ASG established in the present chapter is currently the only available method for long-term, or even semi-permanent, preservation of fish genetic resources. The simplicity and feasibility of this methodology are what enable it to pave the way toward effective and practical conservation of endangered salmonid species and locally endangered salmonid populations, such as bull trout (*Salvelinus confluentus*), sockeye salmon (*Oncorhynchus nerka*) and kunimasu trout (*Oncorhynchus kawamurae*).

[Chapter 2] Although the long-term preservation of fish genetic resources is essential for the conservation of endangered fish species, cryopreservation techniques for fish eggs and embryos are still not developed. A previous study (chapter 1) established a method for harvesting functional eggs and sperm from cryopreserved trout spermatogonia. However, derivation of viable gametes from whole fish that have been frozen without any cryoprotection remains a challenge. If the SSC inside frozen whole fish were capable of differentiating into eggs and sperm in the recipients of different species, then it would be possible to produce fish from frozen extinct fish species by germ cell transplantation into closely related species. Hemizygous p*vasa-Gfp* transgenic rainbow trout were frozen without cryoprotection in a standard deep freezer at -80 ℃. After freezing for up to 3 years, testes isolated from the thawed fish were dissociated into cells. Relatively few of ASG from the frozen-thawed fish remained viable (1,402 ± 251 ASG per fish). However, this proportion did not change significantly with an increase in freezing duration up to 3 years. Frozen-thawed SSC that were intraperitoneally transplanted into wild-type triploid masu salmon migrated toward the recipient gonads where they were incorporated and proliferated rapidly. Although all triploid salmon that did not undergo transplantation were sterile, two out of 12 female recipients (16.7%) and 4 out of 13 male recipients (30.8%) reached sexual maturity at 2 years pt. Eggs and sperm obtained from the recipients were capable of producing normal offspring, with approximately 75% of F1 individuals possessing the *Gfp* gene. These findings suggested that all of the haploid gametes produced by the recipients were derived from the frozen donor fish. Derivation of functional eggs and/or sperm from nonembryonic frozen animals has not yet been achieved in any animal species, as cells within frozen animals tend to lose their functional integrity. The protocol established in the present chapter is thus a breakthrough for complete regeneration of a frozen animal.

Functional eggs and sperm were successfully derived from cryopreserved whole testes (chapter 1) or frozen whole fish (chapter 2) through intraperitoneal transplantation of testicular cells into sterile triploid hatchlings. These two methodologies established from this dissertation would be a powerful tool for long-term preservation of fish genetic resources. Our research group has already started a project to cryopreserve the genetic resources of endangered and commercially valuable fish species, such as kunimasu trout (*Oncorhynchus kawamurae*), Japanese char (*Salvelinus leucomaenis*) and sockeye salmon (*Oncorhynchus nerka*).