Journal of Reproduction and Development, Vol. 52, No. 6, 2006

JSAR Outstanding Presentation Award 2005

Manipulation of Fish Germ Cell: Visualization, Cryopreservation and Transplantation

Tomoyuki OKUTSU[1)], Ayaka YANO[1)], Kazue NAGASAWA[1)], Shinya SHIKINA[1)], Terumasa KOBAYASHI[1)], Yutaka TAKEUCHI[1)] and Goro YOSHIZAKI[1,2)]

[1)]Department of Marine Biosciences, Tokyo University of Marine Science and Technology, 4–5–7 Konan, Minato-ku, Tokyo 108-8477 and [2]SORST, Japan Science and Technology Agency, 4–1–8 Honcho Kawaguchi, Saitama 332-0012, Japan

Abstract. Germ-cell transplantation has many applications in biology and animal husbandry, including investigating the complex processes of germ-cell development and differentiation, producing transgenic animals by genetically modifying germline cells, and creating broodstock systems in which a target species can be produced from a surrogate parent. The germ-cell transplantation technique was initially established in chickens using primordial germ cells (PGCs), and was subsequently extended to mice using spermatogonial stem cells. Recently, we developed the first germ-cell transplantation system in lower vertebrates using fish PGCs and spermatogonia. During mammalian germ-cell transplantation, donor spermatogonial stem cells are introduced into the seminiferous tubules of the recipient testes. By contrast, in the fish germ-cell transplantation system, donor cells are microinjected into the peritoneal cavities of newly hatched embryos; this allows the donor germ cells to migrate towards, and subsequently colonize, the recipient genital ridges. The recipient embryos have immature immune systems, so the donor germ cells can survive and even differentiate into mature gametes in their allogeneic gonads, ultimately leading to the production of normal offspring. In addition, implanted spermatogonia can successfully differentiate into sperm and eggs, respectively, in male and female recipients. The results of transplantation studies in fish are improving our understanding of the development of germ-cell systems during vertebrate evolution.

Key words: Primordial germ cells, Spermatogonial stem cells, Germ cell transplantation, Developmental plasticity, Sexual plasticity

(**J. Reprod. Dev. 52:** 685–693, 2006)

Germline cells, unlike somatic cells, can potentially differentiate into functional gametes and transmit genetic information to subsequent generations via fertilization processes [1]. Primordial germ cells (PGCs) are the first to emerge during germ-cell development, and perform a critical function as the origin of germline cells. In males, PGCs differentiate into spermatogonial stem cells, which have the unique potential to undergo self-renewal and produce daughter cells that can ultimately differentiate into spermatozoa. This process of spermatogenesis can continue throughout the lifetime of male animals. Thus, the transplantation of PGCs and spermatogonial stem cells has potential applications in reproductive medicine, including in infertility treatments. This technique can also function as a powerful tool in transgenic studies, by introducing genetic modifications into animals.

The first PGC transplantation technique to successfully produce donor-derived offspring was

Accepted for publication: August 24, 2006
Correspondence: G. Yoshizaki (e-mail: goro@kaiyodai.ac.jp)

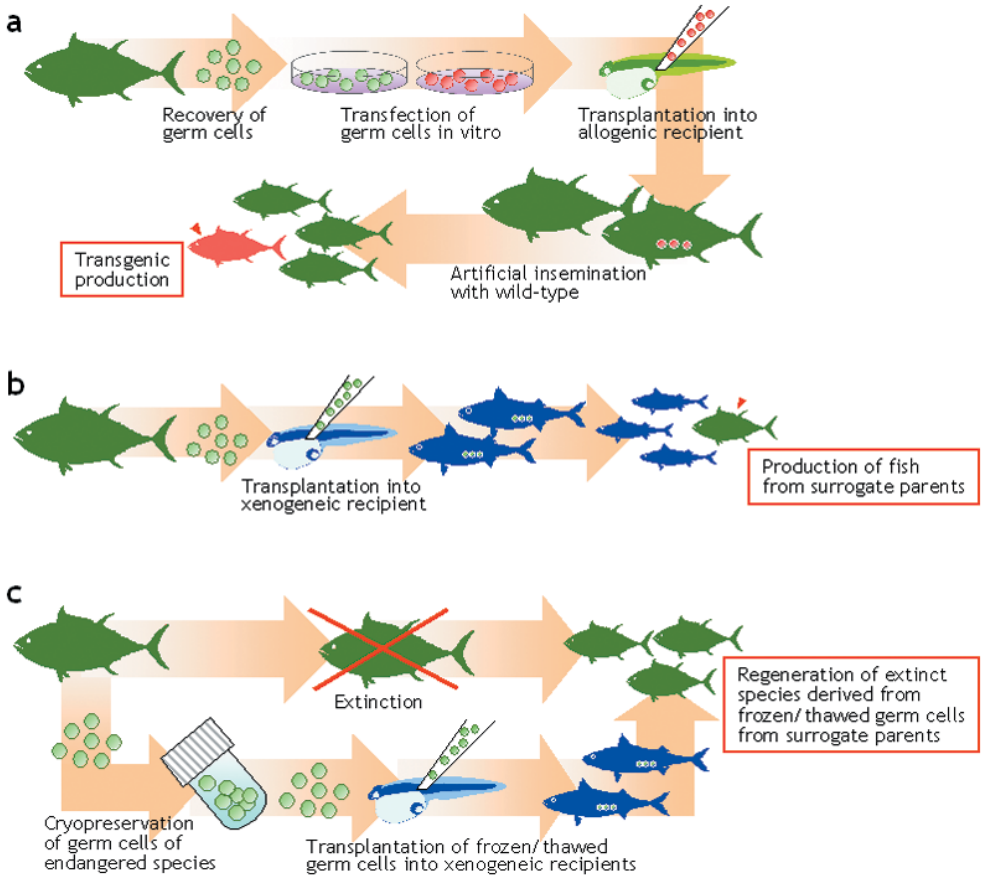

Fig. 1. Applications of fish germ-cell transplantation in the field of bioengineering. (**a**) Transgenic production. This technology can be realized by transplanting germ cells transfected *in vitro* into allogeneic recipients, followed by the retrieval of germ cells. (**b**) Surrogate parent system. Target species can be obtained from the surrogate parents by transplanting the germ cells of the species into xenogeneic recipients. (**c**) Preservation of the genetic resources of endangered species. The cryopreservation and xenogeneic transplantation of germ cells from endangered species make it possible to regenerate them, even in cases of extinction.

established in chickens [2]. In this approach, donor PGCs harvested from the blood stream of early chicken embryos were intravascularly transferred into allogeneic recipients. Subsequently, a spermatogonial transplantation technique was established in mice, in which a testicular cell suspension (including spermatogonial stem cells) was transplanted into the seminiferous tubules of histocompatible recipients [3, 4]. The spermatogonial transplantation approach has been widely employed, as it is the only reliable functional assay for identifying spermatogonial stem cells in mice; these can be distinguished from spermatogonia that are committed to differentiation only by their ability to self-renew, due to the lack of definitive markers for such cells [5]. These advantages have led to the technique being applied to various types of animal, including goats, pigs, cattle and primates [6–9], and most recently to chickens [10, 11].

Previously, germ-cell transplantation techniques were not available for use in lower vertebrates. Consequently, relatively little was known about the function of germ cells in these species. In 2003, we reported on the first PGC transplantation system for fish [12]. In addition, we have recently established a novel system for fish spermatogonial transplantation [13]. These techniques can be used for transplantations between both allogeneic strains [12, 13] and xenogeneic species [14; Okutsu *et al.*,

unpublished data]. These advances have paved the way for innovations in fish bioengineering, such as fish production using transgenic germ cells (Fig. 1a), systems that allow the gametes of economically important large-bodied fish to be obtained from closely related smaller-bodied surrogate species (Fig. 1b), and preservation of the genetic resources of endangered species in combination with the cryopreservation of germ cells (Fig. 1c). The current review outlines our germ-cell transplantation system and discusses future prospects through comparisons with the techniques developed for use in other species.

Visualization of Germline Cells

Before manipulating living germ cells, it is important to visualize them through a non-invasive method. A visualization system employing green fluorescent protein (GFP) was thus identified as an ideal strategy. Germ cells can be labeled with this marker by introducing the *Gfp* gene driven by regulatory regions that are expressed specifically in such cells. A germ cell-specific gene was therefore needed. One candidate gene with suitable characteristics was *vasa*, which is expressed specifically in germ cells in a range of animals from *Drosophila* to humans [15, 16].

The rainbow trout (*Oncorhynchus mykiss*) was identified as a good candidate species, because, as well as being commercially important, its embryos are easily manipulated and dissected owing to their large body size (length at hatching is ~1.5 cm). We therefore cloned and characterized the *vasa* gene from rainbow trout. We subsequently confirmed that the rainbow trout *vasa*-like gene (*RtVLG*) was expressed specifically in germline cells, including PGCs (Fig. 2a) [17–19], spermatogonia (Fig. 2b), oogonia and oocytes (Fig. 2c), using *in situ* hybridization. These results suggested that *RtVLG* could function as a useful germline-cell marker in rainbow trout.

To visualize live germ cells in living embryos, we designed a *Gfp*-expression construct, designated as *vasa-Gfp*, which was under the control of the *RtVLG* regulatory regions. The transgene was constructed using a 4.7-kb 5′ fragment of *RtVLG*, an enhanced *Gfp* gene (Clontech, Palo Alto, CA), a 0.6-kb 3′ untranslated region derived from *RtVLG* complementary DNA (cDNA) and a 1.5-kb 3′ flanking region [18]. Following microinjection into the blastodiscs of fertilized eggs, fluorescent observation of the hatched embryos confirmed that the germline cells of the founder generation showed green fluorescence specifically in the PGCs [18]. Moreover, the subsequent F_1 and F_2 offspring were shown to have *Gfp*-expressing germline cells, including PGCs, spermatogonia, oogonia and oocytes (Fig. 3a–c) [20–22]. Thus, we successfully established a transgenic line in which germ cells could be visually distinguished from somatic cells in living organisms. Viable PGCs [21, 22] and spermatogonia [13] were easily isolated from this transgenic fish species using GFP-dependent flow cytometry. Various transcriptome analyses using PGCs and spermatogonia obtained using this approach are currently advancing our understanding of the molecular mechanisms of germ-cell development.

Establishment of a PGC Transplantation System in Fish

The above-mentioned GFP-labeled germ cells were ideal donor cells for transplantation because their behavior could be traced in recipient embryos through fluorescent observations. Moreover, the GFP fluorescence could be passed on to the next generation. Therefore, donor-derived offspring could easily be distinguished from recipient-derived offspring based on *Gfp* expression in the germ cells.

During the development of our germ-cell transplantation technique for rainbow trout, the most difficult obstacle to overcome was immune rejection of the donor germ cells by the recipients, as we had no inbred or clonal trout strains. Furthermore, we needed a transplantation technique that could be used between allogeneic, and especially xenogeneic, strains, in order to apply this technique to surrogate parent systems for commercially important and endangered fish species (Fig. 1b, c).

To avoid immune rejection, newly hatched embryos were chosen as recipients, because their immune systems were relatively immature [23]. At this stage, the embryos were sexually undifferentiated and contained PGCs in their genital ridges. However, donor cells could not be injected directly into these tissues because of their

small size, unlike the seminiferous tubules commonly used in mammals. To overcome this difficulty, we established a novel transplantation system using the migratory abilities of the PGCs themselves. During early development, the chemokine SDF-1 secreted by the presumptive gonadal regions is known to act as a chemoattractant for migrating PGCs, which express its receptor CXCR4 in zebrafish [24] and mice [25, 26]. In rainbow trout, although the molecular mechanisms of PGC migration were poorly understood, PGCs were known to migrate from the extragonadal areas to the gonadal regions in embryos aged 17–29 days. Subsequently, the PGCs colonized the genital ridges and began to proliferate. Based on these physiological phenomena, we hypothesized that exogenous PGCs transplanted to a site near the endogenous PGC migration pathway might migrate towards the recipient genital ridges, colonize them, and ultimately produce functional gametes (that is, sperm and eggs) that contributed to the recipient germline. To test this hypothesis, we microinjected PGCs isolated from newly hatched embryos carrying *vasa-Gfp* into the peritoneal cavity of allogeneic wild-type embryos at a similar developmental stage (Fig. 4). As predicted, the donor PGCs migrated towards the recipient genital ridges, and were subsequently incorporated into them. We further confirmed that the donor PGCs differentiated synchronously with endogenous germ cells into functional sperm in male recipients and eggs in female recipients. The donor-derived sperm and eggs were shown to be fully functional, as live offspring carrying GFP-labeled germ cells with the donor haplotype were obtained in the F_1 generation by fertilization with wild-type trout gametes [12]. Thus, the transplanted germ cells proliferated and matured normally in an allogeneic microenvironment. These results suggested that immune tolerance was induced in the recipient fish. In support of this hypothesis, no donor PGCs colonized the recipient genital ridges or survived in the allogeneic recipients when they were transplanted into embryos aged ≥45 days, which had more fully developed immune systems [12]. These findings prompted us to perform germ-cell transplantation between xenogeneic species.

Xenogeneic Transplantation of Fish PGCs

As mentioned above, xenogeneic transplantation systems can be used to facilitate the seed production of commercially valuable species. For example, the bluefin tuna (*Thunnus thynnus*) takes 4–5 years to reach sexual maturity, which is difficult to achieve in captivity. Moreover, the body weight of mature bluefin tuna can reach several hundred kilograms. As a result, seed production for this species is expensive in terms of time, cost, labor and space. However, if bluefin tuna PGCs could be transplanted into the mackerel (*Scomber japonicus*), which is a closely related species that reaches maturity in just 2 years at a body weight of ~500 g, bluefin tuna gametes might be more easily and rapidly produced, even in a small fish tank.

In 2004, we reported the establishment of a surrogate parent system in salmonids, by transplanting rainbow trout PGCs into masu salmon (*Oncorhynchus masou*) [14]. The masu salmon is found only in the East Asian Pacific region, whereas the rainbow trout is native to North America. The two species have been phylogenetically separated for ≥8 million years [27]. The most striking biological difference between them is that rainbow trout spawn several times during their life cycle, whereas masu salmon die after their first spawning. In our surrogate system, following transplantation, the donor trout PGCs successfully differentiated into functional spermatozoa in the xenogeneic salmon recipients; donor-derived trout offspring showing normal development were subsequently obtained. These results suggested that the exogenous PGCs were successfully adopted into the xenogeneic microenvironment without immune rejection. Moreover, the surrogate parent system appeared to accelerate the differentiation of donor PGCs into spermatozoa in the xenogeneic recipients, which reached sexual maturation more rapidly than the donor species. Thus, rainbow trout PGCs, which usually took 2 years to differentiate into spermatozoa, reached this stage after just 1 year in the recipient masu salmon—that is, after the intrinsic maturation period for PGCs in the recipient species. In addition to intra-genus germ-cell transplantation (that is, rainbow trout to masu salmon), we recently validated this approach for donor and recipient fish from different genera

within the same family [28; Okutsu *et al.*, unpublished data]. Bluefin tuna and mackerel belong to different genera within the family Scombridae; thus, mackerel could potentially produce gametes derived from transplanted bluefin tuna PGCs using our transplantation system.

Cryopreservation of Fish PGCs

Another application of our transplantation technique is in the conservation of genetic information from species threatened with extinction (Fig. 1c). The cryopreservation of sperm and eggs is one potential method for achieving this goal. However, although sperm cryopreservation methods have been developed for many species, fish eggs have not previously been successfully cryopreserved because of their large size (~0.3–9.5 mm) and high yolk content compared with those of mammals [29]. The only available method for preserving fish genetic resources has thus been maintaining live individuals. However, this approach is vulnerable to disease outbreaks, and to failures of the water and air supplies at fish-rearing facilities. Therefore, the cryopreservation of PGCs, which can eventually be used to generate viable individuals through transplantation into xenogeneic recipients, is an attractive alternative method. We recently reported on the successful cryopreservation and transplantation of trout PGCs, which resulted in the production of functional donor-derived sperm and eggs, and fertile offspring from allogeneic recipients [30]. To date, this is the only reliable method for cryopreserving the complete genetic material of a fish species, including maternally inherited genetic information (such as mitochondrial DNA). The cryopreserved PGCs of endangered species can be regenerated at a later date by transplanting the thawed cells into xenogeneic recipients, even if the donor species has become extinct in the intervening period.

Establishment of a Spermatogonial Transplantation System

Although the techniques described above represented a major breakthrough for fish reproductive technology, the number of PGCs involved was relatively limited (on average ~90 PGCs per newly hatched embryo; Okutsu *et al.*, unpublished data). In addition, the PGCs were accessible only during a short period before gonadal sex differentiation [31]. These factors restricted the applications of the PGC transplantation technique. Spermatogonia were thus identified as an alternative material for overcoming these difficulties, because relatively large numbers are present in the testes at all developmental stages. Furthermore, some spermatogonia act as spermatogonial stem cells, which retain the self-renewal ability essential for clonal expansion, and maintain male fertility throughout an animal's lifespan. Spermatogonia can thus be easily obtained from male fish of any age. These factors suggested that spermatogonial transplantation might be suitable for use in an alternative surrogate parent system, even though spermatogonia were thought to be committed to differentiate into spermatozoa, unlike PGCs that can differentiate into the gametes of both sexes [32]. Based on these considerations, we recently developed a spermatogonial transplantation system in fish [13]. A testicular cell suspension was prepared from *vasa-Gfp* transgenic adult testes by enzymatic digestion. Immunohistochemical analysis with an anti-GFP antibody revealed that only the spermatogonia showed detectable *Gfp* expression in the testes of the *vasa-Gfp* trout (Fig. 5). The suspension, which included ~10,000 *Gfp*-expressing spermatogonia and was predicted to contain a spermatogonial stem-cell population, was intraperitoneally microinjected into newly hatched allogeneic embryos (following the method described for the PGC transplantation). We subsequently followed the behavior of the donor spermatogonia in the recipients based on the green fluorescence. At 20 days post-transplantation (pt), the GFP-labeled germ cells from the adult donors were incorporated into the undifferentiated gonads of the newly hatched allogeneic wild-type recipients (Fig. 6a, b), despite the differences in developmental stage. By 7 months pt, the proliferated donor germ cells had formed a large colony (Fig. 6c, d). At 1 year pt, the spermatogonia-transplanted male recipients had matured, and we performed a progeny test using sperm from recipient males and eggs from wild-type females. The results revealed that donor-derived

spermatozoa were produced in the recipient testes, and were fully functional and capable of yielding live offspring (Fig. 6e, f). In total, 12 of the 26 (46%) male recipients produced donor-derived offspring, confirming that they were germline chimeras [13]. These findings verified that the adult spermatogonia could respond to the cell fate-regulation signals produced by the sexually undifferentiated embryonic gonads. Thus, we concluded that at least a subpopulation of the spermatogonia retained developmental plasticity.

In addition, the donor male germ cells had proliferated and increased their numbers at 2 months pt in the recipient ovaries (Fig. 6g, h), and had even differentiated into oocytes by 7 months pt (Fig. 6i, j). The spermatogonium-derived oocytes developed normally and synchronously with the endogenous oocytes of the recipients. At 2 years pt, the eggs from mature female recipients were artificially inseminated with sperm from intact male trout. The results demonstrated that normal live offspring with the donor-derived haplotype were obtained from 16 of the 40 female recipients (40%; Fig. 6k, l). This suggested that the donor spermatogonia had changed their phenotypic sex and successfully differentiated into fully functional eggs [13].

We recently confirmed that F_1 fish generated from eggs derived from spermatogonia had normal fertility, and were able to produce F_2 offspring (Fig. 6m). In addition, the F_2 generation showed normal development. These results suggest that at least some of the spermatogonia retained both a high level of developmental plasticity and the necessary sexual plasticity to become sperm or eggs. Thus, we concluded that the spermatogonia in adult testes appear to possess a cell population with functional characteristics that are at least partly similar to those of PGCs.

Germ-cell Transplantation Systems in Fish versus Other Animals

As described above, our system allows transplanted donor germ cells to survive and undergo normal gametogenesis in xenogeneic immunocompetent recipients without the need for additional treatments to suppress the immune reaction; this is achieved by using immunoimmature newly hatched embryos as the recipients. By contrast, in mammalian systems designed to transplant germ cells into seminiferous tubules in postnatal recipients, the donor cells cannot survive for long periods in immunocompetent xenogeneic recipients. Although xenogeneic germ-cell transplantation has considerable advantages (as described above), especially for use in domestic animals, such a system has so far only been developed in rodents [6–9]. Moreover, the rodent studies showed that xenogeneic spermatogenesis could only be

Fig. 2. *vasa* RNA localization visualized by *in situ* hybridization. *vasa* RNA was localized in PGCs (**a**), spermatogonia (**b**), and oogonia (arrowhead) and oocytes (arrow) (**c**) in a 45-day-old embryo, 3-month-old testis and 1-year-old ovary, respectively. Panel (**a**) shows a magnified image of a flame in the inset. The scale bars represent 50 μm.

Fig. 3. GFP-expressing PGCs (**a**), spermatogonia in the testes (**b**), and oogonia and oocytes in the ovary (**c**) of a transgenic trout. Scale bars represent 500 μm (**a**) and 5 mm (**b, c**). The boxed area in the schematic drawing corresponds to (**a–c**).

Fig. 4. Intraperitoneal injection of germ cells into newly hatched embryo.

Fig. 5. Immunohistochemistry using anti-GFP antibody of the *vasa-Gfp* transgenic testes. Spermatogonia (asterisks) express intense *Gfp*, but this activity is reduced as they differentiate. The scale bar represents 100 μm.

Fig. 6. Donor spermatogonia kinetics in allogeneic recipients after transplantation, and transmission of the donor haplotype to the offspring. (**a, b**) Incorporation of GFP-labeled donor spermatogonia (arrowheads) into a genital ridge of an allogeneic recipient at 20 days pt in a fluorescent view (**a**), and a magnified image of a flame in a (**b**). (**c, d**) Vast proliferation of donor spermatogonia in an isolated recipient testis at 7 months pt in a bright-field view (**c**) and a fluorescent view (**d**). (**e, f**) Donor spermatogonia-derived F_1 offspring (arrow) showing albino body color obtained from a male recipient (**e**), and their genital ridges containing *Gfp*-labeled germ cells, which is direct evidence of the successful transmission of the donor haplotype (**f**). (**g, h**) Proliferation of donor spermatogonia in a recipient ovary at 2 months pt in a bright-field view (**g**) and a fluorescent view (**h**). (**i, j**) Donor spermatogonia-derived oocytes in a recipient ovary at 7 months pt (**i**) and according to immunohistochemistry using a GFP-specific antibody (**j**). (**k, l**) Donor spermatogonia-derived F_1 offspring (arrow) showing albino body color derived from a female recipient (**k**), and their genital ridges (arrowheads) containing GFP-labeled germ cells (**l**). (**m**) F_2 offspring derived from F_1 fish generated from an egg derived from transplanted spermatogonia. The inset shows the mature F_1 male that produced the F_2 offspring. The scale bars represent 100 μm (**a,b** and **g–j**) and 1 mm (**c, d**).

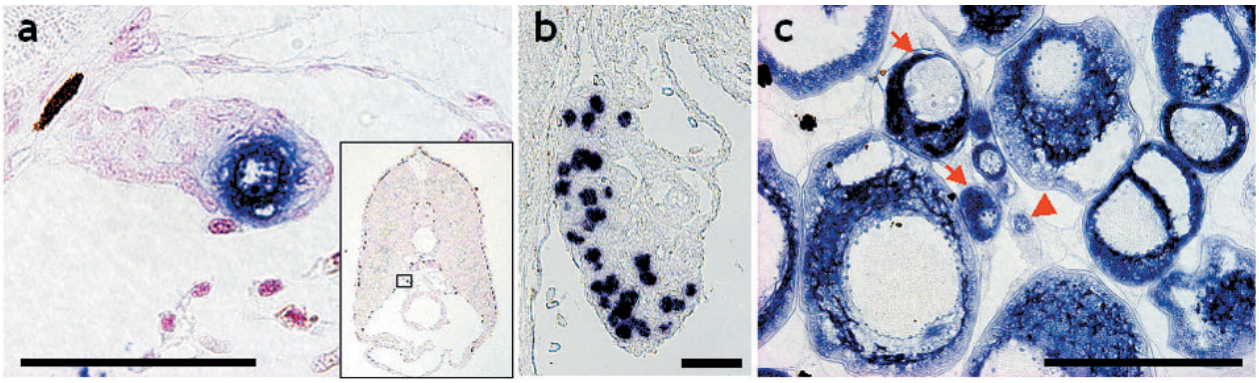

Fig. 2

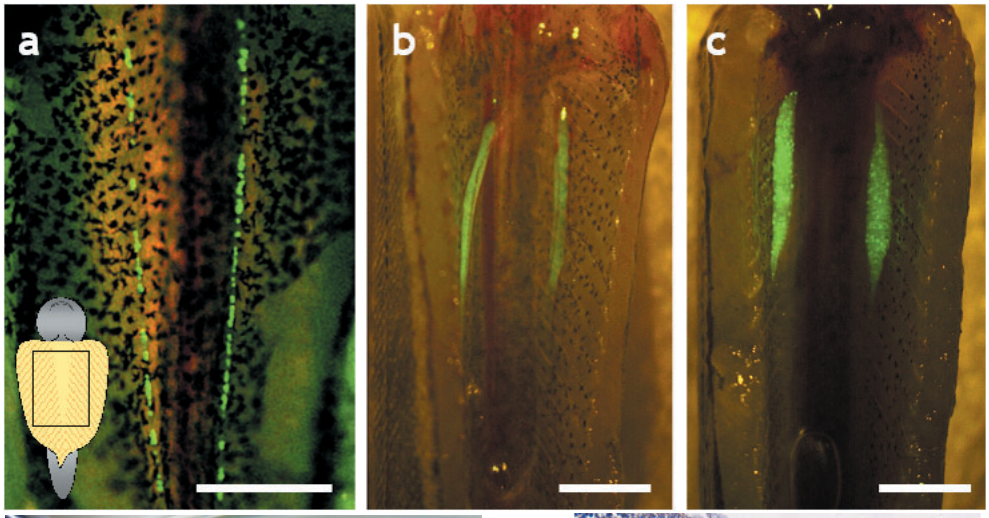

Fig. 3

Fig. 4

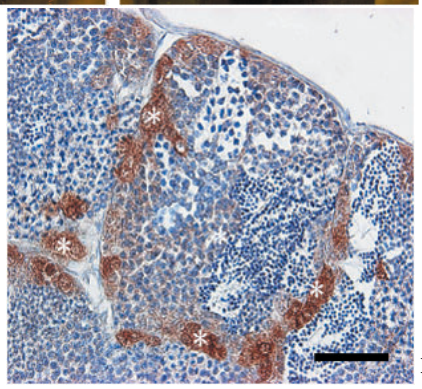

Fig. 5

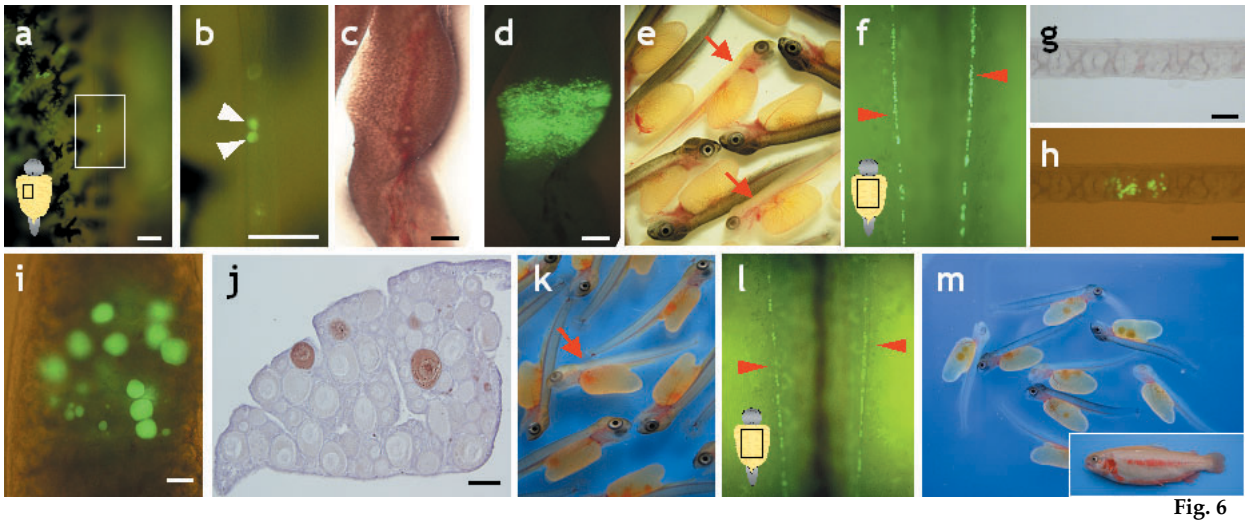

Fig. 6

established if congenitally immunodeficient or immunosuppressed recipients were used [33–35]. Previous findings have highlighted that, when developing a xenogeneic germ-cell transplantation system, it is crucial for the donor germ cells to avoid immune rejection. However, immunodeficient or immunosuppressed recipients have been not available for domestic animals.

One possible alternative approach to establishing xenogeneic transplantation in domestic animals would involve transplanting germ cells into 'prenatal' embryos, in which the immune system has not yet undergone self-education. The self-education process creates a repertoire of lymphocytes that recognize foreign antigens in association with self-major histocompatibility complex antigens. Thus, the introduction of foreign cells into a prenatal embryo prior to the completion of this process could result in donor-specific immune tolerance. This strategy would allow donor germ cells to be retained, and to contribute to the germline of xenogeneic recipients through their lifetime.

To support this proposed approach, a system for transplanting hematopoietic and mesenchymal stem cells into xenogeneic prenatal embryos, known as *in utero* stem-cell transplantation, is available for use in mammals, including domestic animals (reviewed in [36]). A previous review showed that *in utero* stem-cell transplantation could produce donor-specific tolerance for xenogeneic cells, which ultimately resulted in the production of xenogeneic chimerism in hematopoietic cells [36]. Furthermore, as fetuses have smaller numbers of germ cells than newborns, which are commonly used as recipients in mammalian germ-cell transplantation, it should be possible to increase the ratio of transplanted germ cells to endogenous recipient germ cells. This could increase the contribution rate of donor cells to the germline, thereby allowing the recipient to produce sufficient spermatozoa to yield donor-derived offspring through natural mating. Such a technique would be a powerful tool, especially for use in domestic species in which neither *in vitro* fertilization nor intracytoplasmic spermatozoa-injection systems have so far been applied.

As PGC migration towards the presumptive gonadal area is controlled by a similar system in zebrafish [24] and mice [25, 26], intraperitoneal transplantation of germ cells into prenatal embryos, leading to the migration and colonization of donor germ cells in the recipient genital ridges, could be theoretically applied to mammalian species as well as fish. In addition, when differentiated germ cells (such as spermatogonia) are transplanted into a prenatal recipient, genomic imprinting in the donor cells might be erased through intrinsic genomic imprinting by endogenous recipient PGCs; this could shed light on the role of genomic imprinting in germ-cell differentiation and development. Thus, a series of germ-cell transplantation studies in fish, mammals, birds and other animals could facilitate our understanding of the role and development of fundamental germ cell systems during vertebrate evolution.

Acknowledgement

This study was partly supported by Industrial Technology Research Grant Program in 2005 from New Energy and Industrial Technology Development Organization (NEDO) of Japan.

References

1. **Wylie C**. Germ cells. *Cell* 1999; 96: 165–174.
2. **Tajima A, Naito M, Yasuda Y, Kuwana T**. Production of germ line chimera by transfer of primordial germ cells in the domestic chicken (*Gallus domesticus*). *Theriogenology* 1993; 40: 509–519.
3. **Brinster RL, Zimmermann JW**. Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci USA* 1994; 91: 11298–11302.
4. **Brinster RL, Avarbock MR**. Germline transmission of donor haplotype following spermatogonial transplantation. *Proc Natl Acad Sci USA* 1994; 91: 11303–11307.
5. **Brinster RL**. Germline stem cell transplantation and transgenesis. *Science* 2002; 296: 2174–2176.
6. **Schlatt S**. Spermatogonial stem cell preservation and transplantation. *Mol Cell Endocrinol* 2002; 187: 107–111.
7. **Dobrinski I**. Germ cell transplantation. *Semin Reprod Med* 2005; 23: 257–265.
8. **Dobrinski I**. Germ cell transplantation and testis tissue xenografting in domestic animals. *Anim Reprod Sci* 2005; 89: 137–145.

9. **Hill JR, Dobrinski I**. Male germ cell transplantation in livestock. *Reprod Fertil Dev* 2006; 18: 13–18.
10. **Lee YM, Jung JG, Kim JN, Park TS, Kim TM, Shin SS, Kang DK, Lim JM, Han JY**. A testis-mediated germline chimera production based on transfer of chicken testicular cells directly into heterologous testes. *Biol Reprod* 2006 (in press).
11. **Trefil P, Mikalova A, Mucksova J, Hejnar J, Poplstein M, Bakst M, Kalina J, Brillard JP**. Restoration of spermatogenesis and male fertility by transplantation of dispersed testicular cells in the chicken. *Biol Reprod* 2006 (in press).
12. **Takeuchi Y, Yoshizaki G, Takeuchi T**. Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. *Biol Reprod* 2003; 69: 1142–1149.
13. **Okutsu T, Suzuki K, Takeuchi Y, Takeuchi T, Yoshizaki G**. Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. *Proc Natl Acad Sci USA* 2006; 103: 2725–2729.
14. **Takeuchi Y, Yoshizaki G, Takeuchi T**. Surrogate broodstock produces salmonids. *Nature* 2004; 430: 629–630.
15. **Ikenishi K**. Germ plasm in Caenorhabditis elegans, Drosophila and Xenopus. *Dev Growth Differ* 1998; 40: 1–10.
16. **Raz E**. The function and regulation of vasa-like genes in germ-cell development. *Genome Biol* 2000; 1: reviews 1017.1–1017.6.
17. **Yoshizaki G, Sakatani S, Tominaga H, Takeuchi T**. Cloning and characterization of a vasa-like gene in rainbow trout and its expression in the germ cell lineage. *Mol Reprod Dev* 2000; 55: 364–371.
18. **Yoshizaki G, Takeuchi Y, Sakatani S, Takeuchi T**. Germ cell-specific expression of green fluorescent protein in transgenic rainbow trout under control of the rainbow trout vasa-like gene promoter. *Int J Dev Biol* 2000; 44: 323–326.
19. **Yoshizaki G, Takeuchi Y, Kobayashi T, Ihara S, Takeuchi T**. Primordial germ cells: the blueprint for a piscine life. Fish Physiol Biochem 2002; 26: 3–12.
20. **Yoshizaki G, Takeuchi Y, Kobayashi T, Takeuchi T**. Primordial germ cells: a novel tool for fish bioengineering. *Fish Physiol Biochem* 2003; 28: 453–457.
21. **Takeuchi Y, Yoshizaki G, Kobayashi T, Takeuchi T**. Mass isolation of primordial germ cells from transgenic rainbow trout carrying the green fluorescent protein gene driven by the *vasa* gene promoter. *Biol Reprod* 2002; 67: 1087–1092.
22. **Kobayashi T, Yoshizaki G, Takeuchi Y, Takeuchi T**. Isolation of highly pure and viable primordial germ cells from rainbow trout by GFP-dependent flow cytometry. *Mol Reprod Dev* 2004; 67: 91–100.
23. **Manning MJ, Nakanishi T**. The specific immune system: cellular defences. In: Iwama G, Nakanishi T (eds.), The Fish Immune System. New York: Academic Press; 1996: 159–205.
24. **Doitsidou M, Reichman-Fried M, Stebler J, Koprunner M, Dorries J, Meyer D, Esguerra CV, Leung T, Raz E**. Guidance of primordial germ cell migration by the chemokine SDF-1. *Cell* 2002; 111: 647–659.
25. **Ara T, Nakamura Y, Egawa T, Sugiyama T, Abe K, Kishimoto T, Matsui Y, Nagasawa T**. Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). *Proc Natl Acad Sci USA* 2003; 100: 5319–5323.
26. **Molyneaux KA, Zinszner H, Kunwar PS, Schaible K, Stebler J, Sunshine MJ, O'Brien W, Raz E, Littman D, Wylie C, Lehmann R**. The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. *Development* 2003; 130: 4279–4286.
27. **Mackay SJ, Devlin RH, Smith MJ**. Phylogeny of Pacific salmon and trout based on growth hormone type-2 and mitochondrial NADH dehydrogenase subunit 3 DNA sequences. *Can J Fish Aquat Sci* 1996; 53: 1165–1176.
28. **Yoshizaki G, Tago Y, Takeuchi Y, Sawatari E, Kobayashi T, Takeuchi T**. Green fluorescent protein labeling of primordial germ cells using a nontransgenic method and its application for germ cell transplantation in Salmonidae. *Biol Reprod* 2005; 73: 88–93.
29. **Chao NH, Liao IC**. Cryopreservation of finfish and shellfish gametes and embryos. *Aquaculture* 2001; 197: 161–189.
30. **Kobayashi T, Takeuchi Y, Takeuchi T, Yoshizaki G**. Generation of viable fish from cryopreserved primordial germ cells. *Mol Reprod Dev* 2006.
31. **Braat AK, Speksnijder JE, Zivkovic D**. Germ line development in fishes. *Int J Dev Biol* 1999; 43: 745–760.
32. **Devlin RH, Nagahama Y**. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* 2002; 208: 191–364.
33. **Clouthier DE, Avarbock MR, Maika SD, Hammer RE, Brinster RL**. Rat spermatogenesis in mouse testis. *Nature* 1996; 381: 418–421.
34. **Ogawa T, Dobrinski I, Avarbock MR, Brinster RL**. Xenogeneic spermatogenesis following transplantation of hamster germ cells to mouse testes. *Biol Reprod* 1999; 60: 515–521.
35. **Zhang Z, Renfree MB, Short RV**. Successful intra- and interspecific male germ cell transplantation in the rat. *Biol Reprod* 2003; 68: 961–967.
36. **Flake AW**. In utero stem cell transplantation. *Best Pract Res Clin Obstet Gynaecol* 2004; 18: 941–958.