Title: Developmental Biotechnology for Aquaculture, with Special Reference to Surrogate Production in Teleost fishes.

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Abstract

The purpose of this review is to introduce surrogate production as a new technique for fish-seed production in aquaculture. Surrogate production in fish is a technique used to obtain the gametes of a certain genotype through the gonad of another genotype. It is achieved by inducing germ-line chimerism between different species during early development. Primordial germ cells (PGCs) are a key material of this technique to induce germ-line chimera. In several species, it has been reported that PGCs differentiated from the blastomeres inherited some maternally supplied mRNA located at the terminal regions of the early cleavage furrows. PGCs from donor species (or strains) are isolated and transplanted into host species to induce the germ-line chimera. Four methods for inducing germ-line chimera, namely, blastomere transplantation, blastoderm-graft transplantation, transplantation of PGC from the genital ridge and transplantation visualized PGC with GFP fluorescence, are described. Several problems preventing the successful induction of germ-line chimera in various fish species are mentioned. Surrogate production, however, has the possibility to allow efficient fish-seed production and effective breeding and transfer of biodiversity to an aquaculture strain. Conservation and efficient utilization of genetic resources will be achieved through surrogate production combined with the cryopreservation of PGCs.

Keywords: aquaculture, germ-line chimera, primordial germ cell, PGC, surrogate production, teleost

1. Introduction

The expansion of food production has been required in the world due to the increased number of the human population. In agriculture, mechanization, chemical fertilizer and selective breeding have increased food production. Especially, selective breeding, using several methods, raises the crop yields, supporting food consumption of mankind. In recent decades, aquaculture has also shown great advancements, such that about 30% of the world's fish is now supplied by aquaculture (FAO, 2002). The technical procedures in various aspects of effective aquaculture have also been improved. Reproductive biology makes it possible to induce differentiation and maturation of functional gametes for seed production in a large number of fish species, and many fish species are now being cultured under artificially regulated conditions. Chromosome set manipulation techniques make it possible to realize sex-manipulation, sterilization and establishment of inbred stains by cloning (Arai, 1997). Transgenic techniques were also developed to enhance growth (Devlin et al., 1994; Nam et al., 2001, 2002). Genetic infrastructures, such as whole genome sequencing, linkage mapping and EST, are required for further improvement by maker-assisted selection, but at present only exist for a limited number of aquaculture species (Taniguchi, 2000; Coimbra et al., 2003; Ohara et al., 2005). On the one hand, the biodiversity of fish species is still enormous in nature. We must establish more effective systems to utilize natural bio-resources for aquaculture production, by integrating the genetic infrastructure and developmental biotechnology.

Gametes and fertilized eggs have been the major material for developmental technologies, because of their ability to produce the next generation. These cells, however, are special in terms of size, morphology and function, and are difficult to manipulate genetically and cytogenetically. Primordial germ cells (PGCs) are the germ cells before they have arrived at the genital primordium during the embryonic stage, and exhibit a larger size than somatic cells. They are capable of differentiating into functional gametes under the appropriate host condition *in vivo*. Therefore, PGCs are the new materials for developmental biotechnologies for aquaculture, especially for the realization of surrogate production (Yamaha et al., 1999; Yamaha, 2003; Yoshizaki et al., 2003; Saito and Yamaha 2004).

Surrogate production in teleost fish is achieved by inducing germ-line chimeras. To make germ-line chimeras, the PGCs are isolated from donor embryo and transplanted into the host embryos. The hosts become germ-line chimeras if the transplanted PGCs successively migrate to the genital primordium and differentiate into functional gametes. In consequence, donor genotypes can be restored in the next generation. The fish-seed

production is expected to become more efficient by the realization of surrogate production using germ-line chimeras between two different species whose biological properties are different from each other. For example, the life cycle of the fish might be extremely shortened if a species that has shorter life cycle is used as the surrogate parent and produces donor gametes. Moreover, if PGCs from larvae distributed in nature are collected and reproduced through germ-line chimera, we might be able to keep enormous genetic bio-diversity in stock seedlings for marine ranching. In this article, we review the present status of this new biotechnology as well as perspective for aquaculture.

2. Primordial germ cells

2.1. Origin of PGCs

In order to manipulate PGCs for surrogate production, the biological properties of PGCs have to be studied in as many aquaculture species as possible. However, information for teleost species, in which the differentiation of PGCs has been clarified, is still scanty. Until a decade ago, the origin of PGCs was histologically traced back only to the late-blastula or the somitogenesis stage at best, and by morphological characteristics, namely roundish, relatively large cells with large nuclei and a clear nuclear envelope (large mouth black bass, Johnston, 1951; medaka, Gamo, 1961; *Barbus conchonius*, Timmermans and Taverne, 1989). Therefore, it was unknown whether the PGCs differentiate due to maternal factors or to developmental induction.

In 1997, it was shown in zebrafish that PGCs inherited maternally-supplied mRNA located at the terminal regions of the early cleavage furrow (Yoon et al., 1997). The maternal mRNA included the gene homologous to *vasa* required for the formation of polar granules in *Drosophila.* Thereafter, a similar spatial distribution of *vasa* was reported in several fish species (goldfish, Otani et al., 2002; tetra, Fegrade's danio and carp, Knout et al., 2002a; ice goby and ukigori goby, Saito et al., 2004a, b). When the region in which the maternal mRNAs were located was surgically removed at early cleavage stages, no PGCs were formed in the resultant embryo (Hashimoto et al., 2004). In this region of the early cleavage stage, other mRNAs, namely *nos*1 (Köprunner et al., 2001), *dead end* (Weidinger et al., 2003) and *dazl* (Hashimoto et al., 2004), supplied maternally were found and transmitted to the PGCs. These results suggest that teleost fish PGCs are predetermined by maternal factors. On the other hand, there are a few species, such as medaka and rainbow trout, in which the origin of PGCs has not been traced back to the early cleavage stage by *vasa* mRNA localization (Shinomiya et al., 2000; Yoshizaki et al., 2000; Knout et al., 2002a). There are few reports at present about PGCs in important aquaculture

species.

2.2. Migration of PGCs

Some founder cells that have inherited maternal mRNAs as described above proliferate in the course of development from the blastula to gastrula stage and then differentiate into PGCs. They locate around the marginal part of the blastoderm during the blastula stage, and redistribute in the embryo during morphogenetic movement with convergence and extension. In zebrafish, in which the migration process of PGCs has been studied in detail, PGC progenitors localize at the lower border of the blastoderm at the blastula stage, move to the dorsal side along the boundary between trunk and head paraxial mesoderm during epiboly, and form two clusters at the level of the first somite during the early segmentation stage (Olsen et al., 1997; Yoon et al., 1997; Weindinger et al., 1999; Raz and Hopins, 2002). Thereafter, the clusters moved posterior-ward and settled at the junction of yolk extension. PGCs finally settled in the upper part of the body cavity, at the genital ridge, *via* the side of the lateral plate mesoderm and dorsal peritoneum. The migration of PGCs' clusters are integrated by chemokine signals from somatic cells (Doitsidou et al., 2002).

There are several inter-specific differences in PGCs' migration route. In ukigori goby, PGCs do not form any clusters, but align from head to tail bud along both sides of the body (Saito et al., 2004). During the late somitogenesis period, PGCs mainly aggregate just underneath the body axis, and align again along both sides of the gut. Thereafter, PGCs form broad aggregations around the anterior part of yolk extension, and finally settle in the upper part of body cavity, via the surface of the gut. The aggregation of PGCs under the body axis is also observed histologically in ice goby (Saito et al., 2002). In goldfish, PGCs also align in a similar manner, as observed in ukigori, but never move underneath the body before gut formation (Otani et al., 2002). In ukigori goby and goldfish, ectopic PGCs around the head region are frequently observed in normal development (Otani e al., 2002; and Saito et al., 2004a). When the blastoderm was removed and transplanted onto the other blastula, from which the upper part of blastoderm was removed, ectopic PGCs around the head region increased in number (Nagai et al., 2004; Otani et al., 2005). Therefore, ectopic PGCs around the head region are explained by the distribution of its founder around the animal part of the blastoderm at the phase from the blastula to gastrula stage by the heavy mixing of the blastomere during these stages (Otani et al., 2002, 2005; Saito 2002).

When we compared PGCs' migration among species, typical differences were

observed in the following four points; (1) the localization pattern of PGCs at the pre-somite stage, (2) the distribution pattern lateral to the embryonic body during the early-somitogenesis period, (3) the direction of subsequent movement from the lateral position to the embryonic body after the mid-somitogenesis period, and (4) the final localization in antero-posterior axis (Otani et al., 2002; Saito et al., submitted). These results are important for the successful integration of donor PGCs into the host genital ridge.

3. Induction of germ-line chimera

Surrogate production in teleost fishes is achieved by inducing germ-line chimeras. To make germ-line chimeras, the PGCs are isolated and transplanted into the host embryo. In teleost fishes, the following four methods for inducing germ-line chimera have been reported, (1) blastomere transplantation, (2) blastoderm-graft transplantation, (3) transplantation of PGC from the genital ridge, and (4) GFP fluorescence-visualized PGC transplantation. Germ-line chimera is also induced by transplantation of sexually immature gonad into host in rainbow trout (Nagler *et al.,* 2001). Isogenic (cloned) population is required in this transplantation, because of the immunological problem between donor and host individuals. Therefore, we don't take up this transplantation in this article.

3.1. Blastomere transplantation

In this method, donor blastomeres are randomly sucked up with a glass micro-needle and transplanted into host embryo at the blastula stage. In a few experiments, chimeric conditions in the resultant embryos were visualized by the donor-host combination between wild-type and albino strains (zebrafish, Lin et al., 1993; medaka, Wakamatsu et al., 1994, rainbow trout, Takeuchi et al., 2001; loach, Nakagawa et al., 2002). Transplanted donor blastomeres differentiate into many kinds of cells, including germ cells, in each embryo, because both somatic- and germ-line blastomeres are included. It was reported in a cell-lineage and blastomere transplantation study that the blastomeres show pluripotency until the early gastrula stage (Kimmel et al., 1990; Ho and Kimmel, 1993). Therefore, it is considered that the donor blastomeres differentiate according to the fate map at the mid-gastrula stage. In zebrafish, filial wild-type embryos were obtained from the resultant chimeric fish crossed with the albino strain, suggesting that donor (wild-type) blastomeres differentiated functional germ-line cells (Lin et al., 1993). At that time, however, the germ-line lineage in teleost fish had not been analyzed. The frequencies of

germ-line chimeras were less than 32% (Lin et al., 1993; Wakamatsu et al., 1993). Therefore, germ-line chimerism is not always achieved in the resultant embryos, because of the frequent absence of the PGC in donor cells. Thus, the success rate of germ-line chimerism must be increased by other approaches.

3.2. Blastoderm-graft transplantation

In early fish embryos, the blastoderm is highly pluripotent. This property of the blastoderm was shown in the experiment of graft transplantation with a micro-surgical operation in goldfish. When the upper or lower part of the blastoderm was micro-surgically removed, or when an embryo was transplanted with the whole blastoderm from another embryo onto the animal part of the blastoderm, the resultant embryos developed normally and at high frequency (Yamaha et al., 1997, 1998). In the embryos in which the lower part of the blastoderm was removed, the number of PGCs decreased, but never decreased in the embryos in which the upper part was removed (Kazama-Wakabayashi et al., 1999). Therefore, germ-line chimeras are successfully induced by the transplantation of the lower part of the blastoderm (Yamaha et al., 2001; Yamaha et al., 2003; Nagai et al., 2004, 2005).

PGCs differentiate even from an egg fragment. When the fertilized egg is cut horizontally in goldfish, the blastodisc on the resultant animal fragment cleaves normally. Embryos from a half fragment exhibit a series of malformations, such as radial symmetry, acephaly and cyclops or fused eye after epiboly (Mizuno et al., 1997,1999). Many of the one-third fragments at the 1-cell stage and most of the 2-cell blastodiscs at the 2-cell stage developed spherical embryos (Otani et al., 2005). Even in these deformed embryos, PGCs with *vasa* mRNA were detected by *in situ* hybridization (Otani et al., 2005). When the spherical embryo is transplanted onto the animal part of a normal blastula embryo, PGCs derived from a spherical embryo move to the ectopic region around the head and the normal region at the genital primordium in the resultant embryo (Otani et al., 2005). These results suggest that PGCs derived from the small animal fragment are sufficiently functional to move to the genital primordium, and that the PGCs located at the animal part of the blastula are able to move to the genital primordium during subsequent development. Therefore, germ-line chimeras can also be induced by transplantation of the PGCs differentiated in the egg fragment.

3.3. Transplantation of PGCs obtained from the genital ridge

Germ-line chimeras were also induced by blastomere transplantation from the wild strain to the albino strain in rainbow trout (Takeuchi et al., 2001). The operated embryos

survived at 8.0% (19/238) at spawning, and the frequency of germ-line chimeras was 31.6% (6/19). Isolation and transplantation of PGCs should be carried out at more advanced stages of development, because of the low survival after operation.

In rainbow trout, a transgenic strain in which germ line cells are visualized with GFP fluorescence has been established (Takeuchi et al., 2002). PGCs with GFP fluorescence were identified from somatic cells in the genital ridge (Takeuchi et al., 2002). When PGCs were dissociated from the isolated genital ridge at the hatch stage and injected into the peritoneal cavity of normal host embryos, donor PGCs move to the host genital ridge. Individuals that developed from operated embryos had oogonia and oocytes with GFP fluorescence and produced gametes with a transgenic GFP gene, suggesting that the germ-line chimera was successfully induced (Takeuchi et al., 2003). In the transgenic line, PGCs with fluorescence could be selected using a cell sorting apparatus (Kobayashi et al., 2004). When injected, the donor PGCs can move to the host genital ridge, regardless of whether they are sorted or not from dissociated somatic cells (Takeuchi et al., 2003). Because the germ-line chimera could induce about 40% of the embryos operated using this technique, this technique is not always perfect. Moreover, PGCs of the genital ridge from the advanced stage lost their ability to migrate to the host genital ridge, while the condition of host peritoneal cavities for the migration of PGCs seemed to change with the advance of development (Takeuchi et al., 2003). Therefore, the appropriate stages of donor PGCs and recipient embryo should be determined.

3.4. Hetero-chronic transplantation of GFP fluorescence-visualized PGCs

Salmonids have relatively large embryos, so we can remove the genital ridge from the hatched embryo and transplant it into the peritoneal cavity of embryos. Since many fish embryos have small eggs and embryos, extraction of the genital primordium and the subsequent transplantation of PGCs are difficult in most fish species. In fish species in which the embryos are small and fragile, it is also difficult to transplant isolated PGCs to the host embryo. In medaka, transgenic lines with GFP fluorescence in germ cells were established (Tanaka et al., 2001). It is difficult to induce such transgenic lines in many cultured fishes, due to a lack of social acceptance. Therefore, other techniques will be required.

The PGCs in teleost fishes are generally not distinguishable from somatic cells in their vital condition during embryonic development. It is laborious and impracticable to isolate PGCs by producing transgenic strains with GFP-labeled germ cells in rainbow trout. Thus, other methods are required for the detection of live PGCs.

Artificial GFP mRNA combined with the 3'UTR of germ-line specific mRNA, such as *vasa* and *nanos1,* visualized PGCs during embryonic development, when they were injected into the fertilized eggs (Köprunner et al., 2001; Yoshizaki et al., 2005). When the visualized PGCs in the genital primordium of some salmonid fishes were isolated and re-transplanted into the peritoneal cavity, they moved to the genital primordium in the host embryo, inducing germ-line chimera (Yoshizaki et al., 2005). In some cyprinid fishes, when visualized PGCs from the somitogenesis stage were transplanted hetero-chronically into a blastula stage embryo, many moved to genital primordium in the host embryo, while some strayed and arrived at the ectopic region around the head, like the transplantation of graft material, as described above (Saito et al., submitted). PGCs that dissociated before the 20-somite stage moved to the genital ridge in host embryogenesis, while those after that stage mostly did not. This result suggests that PGCs change their properties during development. Therefore, for successful induction of germ-line chimera, it is necessary to consider the developmental stage of the dissociated PGCs in the case of hetero-chronic transplantation.

As a next step for germ-line chimera in fish species, several studies are performed for establishment of culture-cells, such as embryonic stem cell (ES cell), which can differentiate germ-line cells when they introduced into the host embryos (Wakamatsu et al., 1994; Ma et al., 2001; Fan et al., 2004). However, these studies have been performed in exclusive model-species, such as zebrafish and medaka, and still uncertain. Therefore, we make no mention of the germ-line chimera made from culture cells in this review.

4. Problems in the induction of germ-line chimera

Germ-line chimerism has as yet been induced in only a few fish species. Many problems exist before the successful induction of chimerism in various fish species. In this section, we mention several of the predicted problems.

4.1. Chorion

Different types of manipulation, such as injection and transplantation, are required in fish eggs and embryos for the induction of germ line chimera. Fish eggs show diversity in size, specific gravity, adhesion and agglutination (Hirai, 2003). The chorion is a limiting factor for the manipulation of eggs. The chorion of most fish species is tough and difficult remove. Although hatching enzymes secreted from hatching gland cells digest the chorion at the hatching stage, the process of digestion requires considerable time. In

medaka, hatching enzymes collected from just-hatched embryos can be preserved and used for dechorionation (Ishida, 1944a,b). This crude solution contains several factors and the simple component of this digested only one layer of the chorion. In medaka, it is difficult to culture the denuded embryos before epiboly, because of the large yolk cells vulnerable to mechanical damage (Iwamatsu, 1983).

In several cyprinid species, the chorion is easily digested with proteolytic enzymes, such as trypsin and actinase. Treatment of only a proteolytic enzyme solution containing trypsin or actinase adjusted at optimum pH removes the chorion in loach and zebrafish, respectively (Suzuki R. personal communication; Westerfield 1989). In goldfish and common carp, the chorion is easily removed by a solution containing trypsin and urea (Yamaha *et al*., 1986; Ito *et al*., 1999). In these species, denuded embryos are easily cultured under appropriate culture conditions to the hatching stage (Yamaha et al., 1993; Westerfield 1989; Ito et al., 1999; Fujimoto et al., 2004). The treatment with a solution containing trypsin and urea is also effective for softening of the chorion in herring (Saito et al., submitted).

In salmonid species, it was reported that pancreatin solution adjusted to pH 10 can digest the chorion after a short acid treatment at pH 2 for chum salmon (Kanoh and Yamamoto, 1957). However, dechorionation using this treatment is not always successful for several salmon species with tough chorion. In rainbow trout, a solution containing a reduced type of glutathione suppresses the hardening of the chorion after fertilization (Yoshizaki *et al*., 1989). It is also difficult to culture the denuded eggs without mechanically damaging the yolk. Therefore, manipulation, such as the injection of solution and transplantation of blastomeres, was carried out after preventing the hardening without complete dechorionation in salmonid species.

It was reported that the outer opaque layer of the chorion is removed by hypochlorous solution treatment in puffer (Dohtsu, 1986). In northern pike *Esox lucius*, successful dechorionation by protease type XXV was reported, but resultant embryos denuded showed high mortalities (Hallerman *et al.,* 1988). These results collected suggest that the biochemical properties are different among the fish species. Therefore, a method for dechorionation and softening should be developed for each fish species.

In general, denuded eggs before completion of epiboly were fragile for cultivation and micro-operation. It is also required for denuded eggs to set the optimal culture condition. Addition of chicken albmen to culture solution is effective for cultivation and operation of denuded eggs in cyprinid species (Yamaha *et al.,* 1993, 1997, 1998; Fujimoto *et al.,* 2004; Nagai *et al.,* 2005).

4.2. Mass isolation of PGCs

Effective induction of germ-line chimera requires the mass isolation of PGCs from embryos. In rainbow trout, PGCs with GFP-fluorescence were sorted from the transgenic line PGCs by a cell sorting apparatus (Kobayashi et al., 2004). PGCs from a non-transgenic line have been dissociated from the genital primordium collected manually from hatched embryos in salmonid species. In salmonid species, PGCs dissociated from the genital primordium were transplanted into the host embryos without purification, inducing germ-line chimera. It seems to be difficult to collect the genital primordium from small embryos. After visualization of PGCs by injection with GFP-*nos* 1 3'UTR mRNA, PGCs may be sorted using an isolation procedure similar to that used in the PGCs-GFP transgenic line. In many species, in which artificial fertilization is difficult, injection to visualize the PGCs is also difficult at the one-cell stage. For the utilization of PGCs in commercial species, we have to develop improved procedures for the isolation of PGCs. For example, a monoclonal antibody specific to an antigen on the surface of PGCs should be useful. More detailed profiles of PGCs must be disclosed by basic molecular biology.

4.3. Cryopreservation of PGCs

It is very difficult to obtain gametes and embryos of donor and host species at the same time. Therefore, cryopreservation is an essential technique for transplanting PGCs into the host embryo at an appropriate stage. Cryopreserved PGCs are also useful as genetic resources (Kusuda, 2005).

Cryopreservation of the genital ridge with PGCs excised from rainbow trout embryos 30 days after fertilization was reported (Kobayashi et al., 2003). After treatment with the cryoprotectant solution, the genital primordium was frozen and preserved in liquid nitrogen. About 73% of the PGCs survived after thawing. This is a relatively easy and effective method for the cryopreservation of PGCs. However, it seems difficult to obtain the genital primordium from relatively small embryos in non-salmonid fishes.

The cryopreservation of dissociated blastomeres from the blastula was also carried out in several species (Nilsson and Cloud, 1993; Kusuda et al., 2004). The survival of cryopreserved blastomeres was about 50% after thawing (Kusuda et al., 2002, 2004). In goldfish, when blastomeres were transplanted into the host embryo after thawing, a PGC derived from donor blastomeres was detected in the resultant embryo (3%, 1/32) (Kusuda et al., 2004). This low frequency of germ-line chimerism was due to the relatively low number of PGCs per embryo at the blastula stage. The method of

blastomere cryopreservation by Kusuda et al. (2004) may be available for isolated PGCs after the establishment of a mass isolation technique for PGCs.

4.4. Xenogeneic germ-line chimera

In the foregoing section, we mainly summarized knowledge regarding germ-line chimeras between individuals of the same species. Surrogate production, however, through a xenogeneic host with different biological properties, described below, is expected to have the effect of enhancing seed production of the donor, because a xenogeneic host with different properties will support gamete production of the donor. However, it is still unclear whether gametes from one species will be able to differentiate in the other species.

Inter-subspecifically, functional eggs from donor PGCs were produced in goldfish-crucian carp chimeras induced from blastoderm graft transplantation (Yamaha et al., 2001). Functional sperm were also obtained from germ-line chimera induced by the transplantation of goldfish blastoderm grafts to a goldfish by common carp hybrid (Yamaha et al., 2003). In these germ-line chimerae, the development of operated embryos is normal, with a few exceptions. However, it is well known in experimental embryology that the selective cell separation occurs when a plural number of somatic cells derived from different species are mixed. When somatic-line blastomeres are transplanted into the blastoderm from different species, donor cells form aggregates, giving rise to abnormal morphogenesis in subsequent development (Saito, 2004). Hence, blastomere- and blastoderm-transplantation are not promising for a xenogeneic donor and host combination. On the other hand, when PGCs and somatic cells derived from the isolated genital ridge are transplanted into host peritoneal cavities together, the resultant chimeric fish developed normally (Takeuchi et al., 2003). Hence, transplantation of somatic-line cells at the later stage, such as the hatching stage, does not affect the development and survival of the resultant chimeric fish.

Inter-specifically, the sperm of rainbow trout successfully differentiated in the host masu salmon (Takeuchi et al., 2004). This example suggests that donor gametes can differentiate under the host gonadal condition, when similar to that of the donor. It is uncertain whether PGCs transplanted into the host move to the host genital ridge or not. In the transplantation between donor rainbow trout and host masu salmon, about 16.7% (10/60) of donor PGCs were detected in the host gonads (Takeuchi et al., 2004). The frequency is similar to that of intra-species PGCs transplantation in rainbow trout (21.6%, 16/74) (Takeuchi et al., 2003). Therefore, PGCs may migrate under the same mechanism in *Oncorhynchus* species. There are some variations in the migration route and period

among species, as described in section *3.2*. If the migration is mediated by different molecular mechanisms, PGCs could not move to the genital ridge in the host embryo. On the other hand, it was reported in zebrafish that the distribution of cytoplasmic components in PGCs varies during the migration period (Braat et al., 1999; Knout et al., 2000; Nagai et al., 2001). This suggests the possibility that the biochemical properties responsible for migration may change during development. When hetero-chronic transplantation was performed between species with different periods of PGCs migration, PGCs might lose their migration ability to the host genital ridge.

After arrival at the host genital primordium, differentiation of donor PGCs to functional gametes is integrated by host somatic cells. For example, oocytes accumulate the materials of yolk and chorion derived from the host liver through several receptors (Hiramatsu et al., 2002). It is expected that signals and materials derived from the host are not always accepted by the donor germ cells in germ-line chimera. Therefore, it is still unclear in xenogeneic germ-line chimera whether or not the PGCs of the donor differentiate into gametes under the host environment in the donor-host combination of species.

4.5. Establishment of host strain

In the germ-line chimera reported, both the donor and host gametes are spawned simultaneously. For example, the frequencies of gametes derived from donor PGCs were 14.4 to 93.1% in each individual in diploid goldfish-triploid crucian carp chimera induced by blastoderm graft transplantation, in which the number of PGCs from the donor were expected to be equivalent to those from host just after operation (Yamaha *et al*., 2001). In surrogate production, only donor gametes are required. Therefore, we require a sterile host without any gametes from their own germ cells. Probably, the properties of the host must be carefully examined for efficient surrogate production. Sterility and high survival are required in the recipient. Nowadays, we can modify the biological properties by advanced technologies, such as transgenesis and chromosome set manipulation, because we do not use these individuals for food.

In teleosts, considerable numbers of hybrids have been produced and studied in many pairs of different species (Schwartz, 1981). In general, the F1 progeny from an inter-specific cross are sexually abnormal. A bias to one sex is frequently observed and hybrids exhibit a reproductive capacity ranging widely from near-normal fertility to complete sterility in either or both of the sexes. Although the causes of sexual abnormality in F1 progeny are not clear, male sterility was reported in F1 hybrids of the cross between female crucian carp (natural type of goldfish), *Carassius auratus*, and male common carp,

Cyprinus carpio (Makino et al., 1958). In this hybrid, sterility might have resulted from a germ-cell-autonomous abnormality, such as a disturbance of cellular activity and subsequent physiological abnormalities, or the death of spermatocytes during spermatogenesis (Ojima, 1973). Therefore, hybrid fish are good candidates for surrogate parents. When chimerism was induced by transplantation of the lower part of the goldfish blastoderm into the hybrid blastoderm at the blastula stage, motile sperm were obtained from resultant 15 chimeric males by HCG injection, while neither spermatid nor spermatozoa were observed in the testis of the male hybrid (Yamaha et al., 2003). When the sperm of such a chimeric fish was genetically analyzed, only goldfish-specific repetitive DNA sequences were detected. These results revealed that chimeric fish of the cross between a sterile male hybrid and fertile goldfish produced sperm exclusively derived from the donor goldfish. In common carp, a so-called super-male strain, artificially induced by artificial androgenesis of chromosome set manipulation, is available (Kondoh et al., 1989; Kondoh and Satoh, 1990). We were able to produce a sterile male host easily by crossing goldfish (XX) female and common carp super-male (YY). But, when sterile female host are required as surrogate parents, all-female, sterile population must be prepared.

Sterility is induced by auto-triploidization in teleost fish species. Triploid fish induced by chromosome set manipulation inhibiting the second meiotic division have been reported in many aquaculture species (Arai, 1989, 1997, 2001). These artificial triploids may be good candidates for the sterile host of surrogate production. In this context, blastomere transplantation was performed from diploid to triploid rainbow trout (Nilson and Cloud, 1993). But functional gametes from donor diploid were not obtained. The female triploid induced artificially is sterile in crucian carp, *Carassius auratus* (Yamaha and Onozato, 1985).

In zebrafish, sterility is also induced by the inhibition of gene expression required for the maintenance and migration of PGCs. After injecting and anti-sense morpholino oligo nucleotide designed to bind to the *dead end* mRNA into the cytoplasm of a fertilized egg, PGCs lose their way to the genital primordium, thereby inducing sterility without abnormal morphogenesis (Weidinger et al., 2003). Fertility is restored by injecting blastomeres from normal embryos into the blastula developed from the egg injected with the anti-sense morpholino oligo nucleotide (Ciruna et al., 2002). These results suggest that sterile embryos induced using this method should be used for surrogate production. In zebrafish, however, all resultant embryos from such injection develop into male fish, suggesting that artificial sex reversion is required for the induction of female germ-line chimeras (Ciruna et al., 2002). In addition, a morpholino oligo nucleotide has to be

designed specifically for each gene in each fish. This technique also has patent protection. We must therefore pay attention to these points when using morphlino oligo nucleotide. Moreover, the solution has to be injected into the egg cytoplasm in each experiment. This method may be ill-suited to the mass production of germ-line chimera.

Transgenic techniques were developed to enhance growth (Dunham and Devlin, 1998). Transgenic animals themselves are not at present widely accepted as foods. However, transgenic animals may be useful for surrogate parents. For example, two transgenic lines whose filial generation is sterile should be established for the easy acquisition of sterile host embryo. In plants, an approach has been reported whereby modifying plant chloroplasts controls male sterility (Khan, 2005). Such a genetic-engineering system may widen the applicability of surrogate parents.

5. Future development of surrogate production

In agriculture, grafting is the usual technique for preserving established strains without genetic segregation during meiosis, for example, in fruit production, such as apple and grapes. A scion is grafted on the rootstock with different physiological properties from the scion. Grafted individuals are chimeras, because the chimera means biologically an individual comprising two or more kinds of cells with different genotypes. Successful combination of grafting between rootstock and scion follows various qualitative and/or quantitative improvements in the resultant chimeric plant (Neilson-Jones, 1969). For example, dwarfing rootstock prevents the growth of scion, minimizing the height of tree, decreasing plant spacing and increasing the productivity of fruits per unit area. Rootstocks with disease or salt resistance prevent the spread of deadly diseases from the soil to scion, or protect against damage from saltwater, respectively. Some kinds of rootstock improve fruit quality, such as sugar concentration and external appearance. In this context, we use the two genetic properties from the rootstock and scion in the plant chimera. In surrogate production, we may adopt the concept of grafting from plant breeding. In other words, if the gametes are produced from the germ-line chimera, in which PGCs were transplanted into the host with different physiological properties from those of PGCs, we will establish the applicable technology as follows.

5.1. Preservation of the mutant strain

In a model fish, such as medaka or zebrafish, an innumerable number of mutants have been induced in order to analyze the function of genes. The mutant strains presently are preserved as cryopreserved sperm. It takes the period of one generation to recover

the mutant individuals with the desired phenotype from the cryopreserved sperm, while two cycles of generation are necessary in the maternal-effect mutant (Mullins *et al*., 1994: Dosch *et al*., 2004). In strains with recessive mutation, just one-forth individuals with the mutant phenotype are obtained from the crossing of the parent fish with the heterozygous genotype. PGCs replacement between the maternal-zygotic mutant and wild-type was carried out in zebrafish (Ciruna *et al*., 2002). If mutant PGCs are easily isolated and cryopreserved, we can obtain all-mutant embryos through surrogate parents. This procedure reduces the laborious work for maintenance and analyses of mutants.

5.2. Preservation and recovery of endangered-species

Due to increasing human activity, the survival of many wild populations is threatened. Teleost fishes are not an exception. The genetic diversity of wild populations must be preserved for the welfares as well as for the wise use of the gene resources accumulated during the history of earth for future generations. In teleost species, pedigree preservation and sperm cryopreservation have been carried out in a relatively large number of species (Leung and Jamieson, 1991, Kusuda, 2005). However, genetic diversity decreases during pedigree preservation, and maternal genes are not preserved in sperm cryopreservation (Kusuda *et a*l., 2004). Cryopreservation of PGCs and the recovery of species through surrogate parents of related species are useful for the preservation of genetic diversity, including cytoplasmic genes (Kobayashi *et al*., 2003: Yoshizaki *et al*., 2003). However, this strategy must be used as an auxiliary technical method because the isolation of PGCs is expected to be difficult in endangered species. As for the next new innovation, the artificial induction of PGCs may be required.

5.3. Fish-seed with high genetic variation

Single individuals produce gametes with combinatorial genetic variety from their diploid genome. If several PGCs with different genetic backgrounds or lineages are transplanted into the host, the resultant single individual is expected to produce gametes with a wide variety of genetic diversity from donor PGCs. There is the chance that wide genetic varieties are produced from a small number of surrogate parents to which plural numbers of PGCs with genetic variation are transplanted. If PGCs are collected from the embryos of the wild population, wide genetic diversity may be available for basic and applied fisheries science, as described below. These populations with genetic varieties may be suitable for fish-seed for stock enhancement, or for genetic evaluation for a population adapted to aquaculture.

In surrogate production, we have to be more concerned about the frequency of both sexes in fish-seed. Fish-seed from surrogate parents could cause genetic disturbance of sex in the wild population. Fish utilize a wide array of mechanisms to control sex determination (Devlin and Nagahama, 2002). Under some sex determination systems, the even genetic sex is easily reversed by environmental conditions, and an abnormal genotype, such as YY, might be expected after crossing between surrogate parents. The gametes with reversed sex genotype could distort the sex distribution in the wild population. For example, in goldfish with XY sex determination system, when PGCs from fertilization between genetically XX-female and XX- pseudomale goldfish were transplanted into genetically XY goldfish and a common carp hybrid, PGCs differentiated functional sperm (Yamaha et al., 2003). A similar sex reversal of the transplanted PGCs was observed in the germ-line chimera of medaka (Shinomiya et al., 2002). In these species, sex reversal of PGC seems to be easily induced. Therefore, we should carefully consider the risks about fish-seed produced by surrogate production in fish with unknown sex determination system.

5.4. Efficient fish-seed production

Teleost fishes show a variety of reproductive traits, such as maturation size, generation time and fecundity. Artificial fish-seed production is severely restricted by the reproductive traits of the target species. In tuna, fertilized eggs spawned naturally are collected in the open field by an ichthyoplankton net. Artificial maturation and fertilization are nearly impossible, because of its maturation size of about 110 cm in fork length and 21.3 kg in weight in cultured female individuals (Miyashita et al., 2000). Even in ornamental carp propagated in Japan, farmers avoid artificial manipulation, such as hormonal injection to the parent fish, because of the fragility of their skin. When injected experimentally, large amount of hormones are required to induce artificial ovulation. If the gametes of these fish with large maturation sizes are able to differentiate in other fish with a small maturation size, such as goldfish, we will save the space for farming, the time for maturation and money for fish feed. It is also anticipated that farmers can get off their laborious work for fish-seed production in large, valued fish. On the other hand, if the gametes of fish with low fecundity differentiate in other fish with higher fecundity, we would be able to obtain sufficient amounts of eggs at a time. Of course, we should perform the cost and benefit analysis to apply the surrogate production for seed production in these species.

5.4. Effective breeding

In teleost fishes, some fish spawn once in their lifetime, while others do so several times. For example, masu salmon (*Oncorhynchus masou*) and chum salmon (*Oncorhynchus keta*) spawn once per lifetime. If the fish-seed from these species were obtained several times from their surrogate parents, such as rainbow trout, in which gametes are obtained for several years, we could more easily improve these fish genetically.

Many fish species are now being cultured under artificially regulated conditions, but still many commercial fish remain undomesticated. It is not, however, realistic to regulate reproduction in all commercial fishes. If certain species, in which artificial regulation of maturation, chromosome set manipulation and transgenic technology are available, could be used as a host species, many uncultured species would be easily propagated through the technique of surrogate production.

If the age at maturation is shortened through surrogate production, we can rapidly improve the genetic characteristics in some fish species. Namely, if the gametes from fish with a long generation time are produced through surrogate parents with short generation time, a selected strain with superior traits could be selected in a short time. For example, disease resistance is a most desirable character for aquaculture. After mass mortality by infectious disease, fish-seed with resistance should be supplied rapidly. If we can get the PGCs from the offspring of the survivor, in which genes for disease resistance are expected, after mass mortality, we could easily increase the number of fish-seed with disease tolerance. Transgenic techniques were developed to enhance growth in several fish species (Devlin et al., 1994; Nam et al., 2001, 2002). If the generation time is shortened, these transgenic lines are useful candidates for surrogate parents for rapid propagation and breeding of fish-seed. On the other hand, if the direct transgenesis is successfully performed to the isolated PGCs we may get easily transgenic line (Yoshizaki et al., 2003).

5.5. Production of a disease-susceptible strain through the resistant strain

As described in the previous section, fish diseases are a serious problem in aquaculture. A large number of gene resources suitable for aquaculture are lost after mass mortality due to infectious diseases. The establishment of strains with disease resistance is one of the most important goals of fish breeding. However, genetic stocks, including a large variety of useful genes for aquaculture, other than disease resistance, frequently have been lost after accidental mass mortality caused by diseases. Parent fish for fish-seed production might be cultured under separate conditions from the population for

growth enhancement. There are many cases where it is difficult for aquaculture in the open sea to separate the parent fish population from the cultured population. If the several strains of PGCs are transplanted into the host with disease resistance, we should be able to save the valuable gene stock from the accidental mass mortality caused by diseases. Resistance to infectious pancreatic necrosis (IHN) has been reported in rainbow trout (Okamoto et al., 1993). It is also known that certain hybrid salmonids have disease resistance (Chevassus and Dorson, 1990). Several hybrid fishes are also known to be sterile. Therefore, the surrogate system, using sterile hybrid as parents, would seem to be useful for fish-seed production under culture-conditions spreading infectious diseases, avoiding accidental mass mortality. It is also necessary in the host strain to boost immunity by transgenic technology. Actually, several studies show that disease resistances are improved by transgenesis (Dunham et al., 2002; Mao et al., 2004). Genes of disease resistance should be searched in the wild populations for selective breeding.

5.6. Transfer of biodiversity from wild population to aquaculture strain

Reproductive biology makes it possible to induce the differentiation and maturation of functional gametes for seed production in a large number of fish species. Genetic diversity is relatively low among gametes from a limited number of parent fish. At present, we still have a wild population with wide genetic diversity for many species. The morphological characteristics and natural distribution of the early stages of fishes have been studied in Japan (Okiyama, 1988). If PGCs are collected from the natural population by ichthyoplanktonic sampling and functional gametes are produced through surrogate parents, we would be able to get the wide variety of the first filial generation from which the strains with useful genes, for example those of disease resistances, could be selected for aquaculture. We might improve the aquaculture population genetically by crossing between the culture population and gametes from surrogate parents. Fish larvae are collected for stock assessment, and their breeding season and distribution have been gradually disclosed by resource analysis. These data would be available for the collection of PGCs and subsequent surrogate production.

5.7. Induction of diploid gametes

In some teleost species, natural polyploid populations are found. Artificial polyploids also have been induced by chromosome set manipulation (Arai, 1997, 2001). These observations suggest the possibility that polyploid breeding in aquaculture will be available. Triploid individuals have been commercialized in several aquaculture species,

such as salmonids and oysters. However, there have been few reports about the induction of auto- and allo-tetraploidy until now (reviewed by Arai, 1997). Tetraploidy was induced in the limited number of fish species, such as rainbow trout (Chourrout, 1984), but not many in all species studied. For example, in masu salmon, *Oncorhynchus masou,* tetraploid individuals suffer from anemia with a pale body color and died within several days after hatching (Sakao et al., in press). It is considered that tetraploidy itself gives rise to lethality in this species, because gynogenetic diploid individuals induced by the same treatment in the same batch can survive (Sakao et al., in press). Tetraploid individuals destined to die have PGCs in the genital ridge. If tetraploid PGCs are isolated and transplanted into the host, diploid gametes might be obtained through surrogate production. In addition, cell technology to isolate PGCs followed by the induction of tetraploidy by cell-fusion, opens other possibilities of polyploid breeding in teleost fishes.

6. Application of surrogate propagation to flatfish aquaculture

Seedling production for flatfishes has been carried out in 11 species of 8 genera in Japan (Howell and Yamashita, 2005). Sperm in seven species has been cryopreserved using pellet or straw methods (Saito, 1996). Several hybrid stocks have been induced by artificial fertilization between flatfishes (Schwartz, 1981; Liewes, 1984). Artificial chromosome set manipulation and subsequent sex-manipulation were performed in Japanese flounder, *Paralichys olivaceus* (Yamamoto, 1999). In addition, a project involving linkage mapping in Japanese flounder is in progress (Coinbra et al., 2003). Over one hundred flatfish species are distributed commercially (Japan Marine Products Importers Association, 2000). If the commercial species of flatfish whose gametes are not yet induced by artificial regulation of reproduction are propagated through surrogate parents, many flatfish will be come available for aquaculture. Visualization of PGCs has been successfully performed in several aquacultured flatfishes (Goto-Kazeto et al., unpublished data). There is even a report of a sterile hybrid between plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*) (Purdom, 1972). Therefore, all the tools have already been prepared for surrogate production in flatfishes. Moreover, the spatial distribution of fertilized egg and larvae has been examined to elucidate the early life history (Nakagami et al., 2001; Hasegawa et al., 2003; Imura et al., 2004). If the PGCs are sorted by cell sorting apparatus from the eggs and larvae collected from the natural population, we can use natural gene recourses for artificial breeding in flatfish.

7. Conclusion

Aquaculture species are more numeral than land animals, such as cows, pigs, sheep and chickens, in animal husbandry. However, there are a fewer species improved genetically among teleost fishes than in land animals. On the other hand, in fish species, wild populations with wide genetic diversity remain without artificial selection. This suggests that aquaculture species have a large potential for breeding to enhance the productivity of food. The surrogate production of fish, like grafting technology in plant breeding, is regarded as a useful technology for the preservation and effective use of genetic diversity, fish-seed production, and selective breeding.

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Figure legend

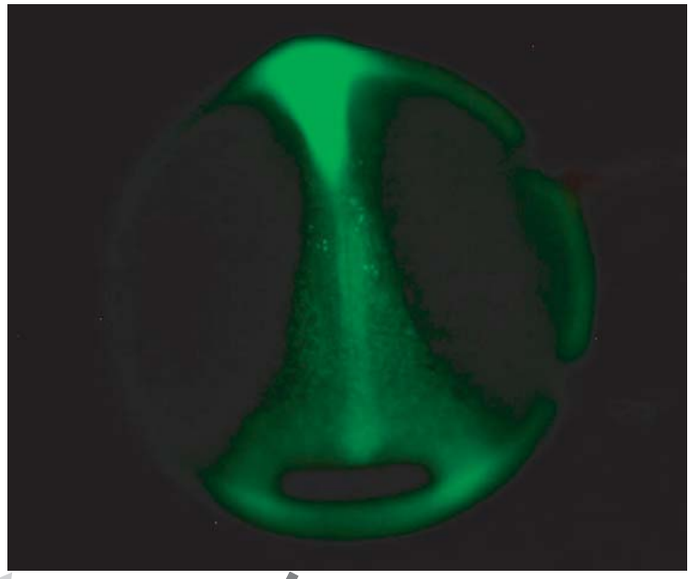
Fig. 1. Schematic illustration of surrogate production of teleost fish. Visualized GFP-labeled primordial germ cells are transplanted into host blastula to induce germ-line chemera (see *section 3.4*).

Donor species

♂ ♀

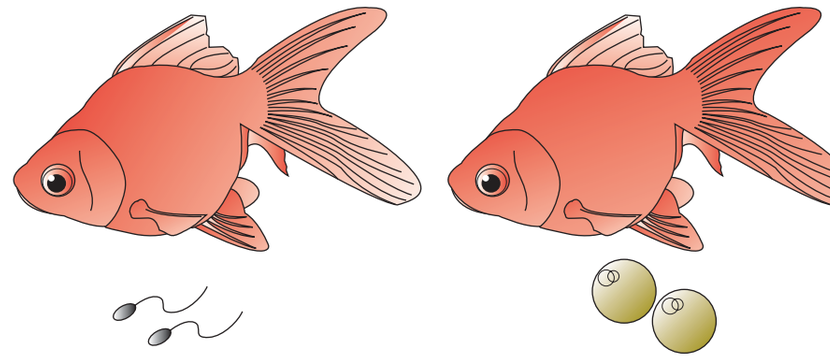
Fertilization

Injection of
GFP-*nos*1-3'UTR
mRNA



Visualization of PGCs
in barfin flounder

Host species

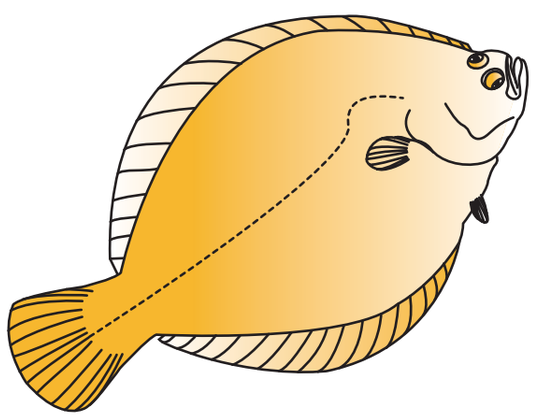


Dissociation of
blastomeres

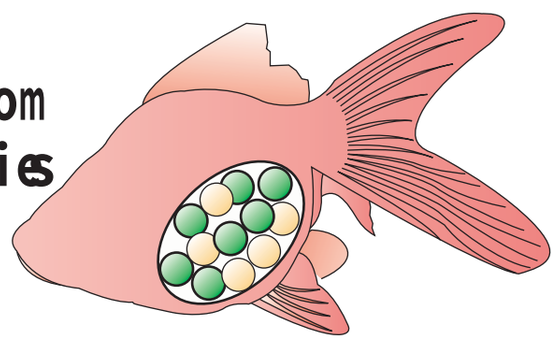
Separation of PGCs

Fertilization
(Sterilization)

Regeneration of donor species



Gametes from
donor species



Germ-line chimera

Transplantation
of PGC