A state-of-the-art review of surrogate propagation in fish

Rie Goto* and Taiju Saito

South Ehime Fisheries Research Center, Ehime University, Ehime, Japan 798-4206

* Corresponding author

Rie Goto, PhD.

Phone: +81 895 73 7112

Fax: +81 895 73 7111

Email: goto.rie.me@ehime-u.ac.jp, riegoto0419@gmail.com

ABSTRACT

Surrogate propagation is a systematic approach to producing donor-derived gametes using germline chimeras. In fish, the use of germline chimeras to study the development of germ cells was first conducted in the 1990s in the model fish species medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). More recently, surrogate propagation has been actively investigated as a means of efficient gamete production not only in model fish species but also in aquaculture species and endangered species. Surrogate propagation has the following components: combination of the donor and host species, host sterilization, donor cell preparation, transplantation of germ cells, and gametogenesis and gamete production in surrogate fish. In this review, we first provide a general overview of previous studies related to germ cell transplantation and the methodologies developed for different species, and consider how these have been applied in practice. Second, we consider the development of primordial germ cells in fish embryos, particularly the molecular biological approaches used for the visualization of germ cells and sterilization of host embryos. Finally, we discuss sex control in germline chimeras, which may be a key component of the use of surrogate production in aquaculture. We focus on techniques to produce sterile fish, as these are crucial to the exclusive production of donor gametes in a surrogate host. The advantages and disadvantages of various aspects of surrogate propagation are discussed, the potential use of surrogate propagation as a seedling production system is considered, and future perspectives for aquaculture are suggested.

Key words: germline chimera, surrogate propagation, chimera, transplantation

1. Introduction

Genetic resources conservation in fish has been mainly conducted through sperm cryopreservation. Fish eggs contain yolk granules as nutrients for embryo development, but the fairly large size of eggs contributes to difficulties in cryopreservation. Reviving species by sperm is partial because mitochondrial DNA is only inherited from the eggs, whereas surrogate propagation can regenerate a complete species from cryopreserved gonads or germ cells. Surrogate propagation is therefore a promising technique to regenerate the genetic traits of endangered and commercially valuable species. Since surrogate propagation in model fishes and some aquaculture species have been extensively studied over the past two decades, knowledge in this area has continuously improved. These efforts are beginning to pay off in the form of practical applications at present (e.g., seedling production of *Takifugu rubripes* using the *T. niphobles* host for farming in Japan and the

production of surrogate parents for the regeneration of huge endangered sturgeons using small-size sturgeon, sterlet (*Acipenser ruthenus*), in the Czech Republic, which will be discussed later). Although the positive aspects of this new technique have been investigated in several papers and reviews, there are many technical and biological limitations faced in surrogate propagation, which include the type of egg used, chorion, size of embryos, and the gene database that can be used for developing molecular tools. Additionally, the availability of usable host species and the skill required to propagate and effective sterilization of the host species are other limitations currently being faced by scientists attempting surrogate propagation. Because the techniques proposed in previous studies to induce germline chimeras are complicated and require delicate and technical manipulation, appropriate methods need to be developed and optimized before applying them to a target species. In this review, we overview the development of methods for the production of germline chimeras from research performed in other animals prior to the establishment of surrogate production in fish. We discuss the techniques developed in fish species and their advantages and disadvantages for obtaining donor-derived gametes; we also as provide practical examples of xenogeneic and allogeneic germline chimeras that have already been discussed in existing literature. We summarize the early development of germ cells, especially during embryonic development, to deepen the understanding of the biological basis of surrogate propagation. Finally, we pose a question on the current situation of surrogate propagation in terms of risk management and present the potential of the effective utilization of fish surrogacy for future aquaculture and genetic resources conservation in fish.

2. Surrogate production in other organisms

The term *grafting* is used to describe the technique of connecting two individual plants into a single individual, and it is widely used in many practical applications in agriculture [1]. In biological terms, chimerism is synonymous to grafting in animals. In animals, germline chimeras, which have germ cells of a genetically different donor in the gonad, can be used to generate offspring derived from the donor cells. As with grafting in plants in agriculture, the use of germline chimeras has great potential in aquaculture applications. For example, germline chimeras can be produced between species or strains with different characteristics: 1) the gametes of a large fish

species might be produced in a smaller species, thereby requiring less space; 2) the gametes of a species that have a long maturation period can be produced in species with a shorter period for producing gametes; 3) when the gene bank for donor gamete production becomes available, the subsequent regeneration of the donor species will occur.

In animals, transplantation techniques have been developed to produce chimeras for use in studies on embryonic development and developmental biology. For example, Spemann and Schotté (1932) transferred embryonic tissues between frogs and salamanders to study the induction ability of these tissues [2]. Similarly, Le Douarin and Teillet (1973) transferred quail and chicken cells during embryonic development to take advantage of their differences in staining properties in histological sections as a means to trace the positions of the transplanted cells [3]. In Drosophila, Technau and Campos-Ortega (1986) produced germline chimeras to study the developmental fate of pole cells [4]. Similar experiments were also performed in frog [5,6]. The objective of Blackler's (1962) study was to examine germline development; to this end, he transplanted migrating primordial germ cells (PGCs) by exchanging the endodermal regions of *Xenopus laevis* and *Xenopus victorianus*. The experiment confirmed the germline transmission of transplanted cells to the next generation.

More recently, reproductive engineering using interspecific chimeras has been conducted in mammals. Fehilly et al. (1984) produced sheep–goat chimeras by fusing early-stage embryos to overcome the reproductive barrier between sheep and goats [7]. Germline chimeras have been produced in mammals to investigate gamete production. For example, rat or hamster spermatogonia have been transplanted to the seminiferous tubules of sterilized male nude mice that have defective T cell functions, including xenograft rejection; analysis of the mice shows that donor gametes are generated in the xenogeneic host following the completion of the entire process of germ cell proliferation and differentiation [8-10]. This approach has also been extended to other mammalian species, such as pig, goat, and rhesus monkey [11-13]. Primordial-stage follicles from mice have also been demonstrated to develop under the support of rat granulosa cells, and they are capable of differentiating into functional oocytes in the xenogeneic environment of the rat ovary. Moreover, these oocytes can be fertilized and can start embryonic development normally [14].

In chicken, PGCs can be isolated during embryonic development from the blood or gonadal tissues [15,16]. Transplantation of isolated PGCs into host embryos can generate germline

chimeras, even between different species. To date, many studies have been performed to improve reproduction in birds, with a particular focus on poultry. Xenogeneic germline transmission has been confirmed across genera (common pheasant to chicken), families (chicken to guinea fowl), and orders (duck to chicken, and houbara bustard [*Chlamydotis undulata*] to chicken). However, a successful transmission has only been accomplished in males; the complexity of oogenesis and the incompatibility between species may have precluded success in females (reviewed by [17]).

3. Surrogate production in fish

Germ cell transplantation studies have been performed in fish species with the aim of improving aquaculture productivity through surrogate production. Several transplantation methods have been developed and tested for the production of germline chimeras in fish (Figure 1). These are described below.

3.1. Germ cell transplantation methods based on experimental embryology

In fish, germline chimeras were first reported in zebrafish. In this study, blastomeres of wild-type zebrafish were randomly aspirated into a glass needle, as the location of the germplasm in blastula-stage embryos were not yet established (Figure 1A). The blastomeres were then transplanted into the host embryos of albino zebrafish at the same developmental stage. As a consequence of the uncertainty of the location of the germplasm, PGCs plus somatic cells were transplanted into the embryos. Donor-derived germ cell transmission to the next generation was evaluated by the presence of pigmentation in offspring. A similar approach was applied in studies using medaka [18], trout (*Oncorhynchus mykiss*)[19], loach (*Misgurnus anguillicaudatus*)[20], and pikeperch (*Sander lucioperca*) [21]. Blastomere transplantation (BT) was also used to generate *maternal-zygotic mutants* in zebrafish [22] and medaka [23]. However, the efficiency of donor germ cell transfer was low and varied among experiments: 11.9%–17.9% in zebrafish, 29.9% in medaka, 31.6% in trout, and 25% in loach and pikeperch. Recently, Li et al. (2016) optimized the BT technique in medaka by using PGC-boosted donors and sterile hosts, and they were able to improve efficiency to nearly 100% [24]. Yoon et al. (1997) successfully identified the location of PGCs in zebrafish by using

whole-mount in situ hybridization (WISH) with *vasa* transcripts [25]. This analysis indicated that PGCs are located at the marginal region of the blastodisc at the blastula stage. Using this information, Yamaha et al. (2001) successfully transferred the PGC-containing lower part of the blastodisc of a donor embryo into a host blastula-stage embryo (blastoderm transplantation [BdT]) to improve the efficiency of PGC transfer [26]. The technique used in this study was originally developed for examining patterning during fish embryonic development [27,28]. The methodology used by Yamaha et al. (2001) yielded landmark results in the field of fish surrogate production, as it enabled the production of xenogeneic fertile germline chimeras between crusian carp (*Carassius carassius*) and goldfish (*Carassius auratus*) [26]. The BdT approach gave donor-derived gametes from all chimeras analyzed. Using the BdT technique with sterile hybrid recipients, Yamaha et al. (2003) confirmed mono-sex and donor-sole gamete production [29]. In this study, goldfish PGC-rich blastula fragments (genetically female, XX) were transplanted into sterile male (XY) hybrid embryos from a cross between female (XX) goldfish and super-male (YY) common carp; donor-derived sperm was produced following maturation of the transplanted embryos. The donor goldfish PGCs (XX) underwent sex reversal and differentiated into sperm in the male hybrids. However, the BT and BdT methodologies cannot be used to produce germline chimeras when the donor and host species are distantly related. Saito et al. (2010) demonstrated that zebrafish embryos transplanted with goldfish or loach (*Misgurnus anguillicaudatus*) blastomeres showed severe malformations because of donor cell aggregation; this effect may be due to differences in cell affinity between donor and host cells [30]. To overcome this problem, Saito et al. (2008) labeled PGCs by injecting GFP-*nos3* 3'UTR mRNA into donor eggs, which dissociated embryonic tissues with enzymatic digestion, and then selected and transplanted a single PGC into a sterilized host embryo under a fluorescence stereomicroscope [31]. With this single PGC transplantation (SPT) approach, germline chimeras were produced between unrelated species—for example, pearl danio to zebrafish—and between different genera (goldfish to zebrafish) and families (loach to zebrafish). Thus, a transplanted PGC from goldfish and loach could migrate to the genital ridge of zebrafish embryos without inducing any malformations. These xenogeneic germline chimeras also developed normally with high PGC transfer efficiency (more than 40%). Moreover, donor-derived sperm was obtained in all combinations tested. Interestingly, a single PGC was sufficient for gonad formation and gametogenesis when transplanted into a sterile host. Similar transplantation experiments have since been performed for many fish combinations with a variety of phylogenetic relationships [32,33]. Moreover, the SPT method can provide experimentally a unique condition in which each gonad has germ cells originating from different donors (Fig. 2). A summary of these experiments is presented

in Table 1. This information will benefit researchers in terms of understanding how phylogenetic differences affect surrogate propagation in fish. However, the methodologies have some limitations. BT, BdT, and SPT involve dechorionation of eggs and require expertise in micromanipulation. PGC transplantation using these methods must be undertaken during a short window in embryonic development, and it can only be used for species for which embryonic manipulation techniques have been developed, such as model fish species (e.g., zebrafish, medaka, goldfish, and loach). However, as such techniques are not available for most marine fish species, PGC transplantation cannot be utilized; this is particularly the case for species in which dechorionation and embryonic manipulation are extremely difficult. Nevertheless, BT, BdT, and SPT offer the advantage of traceability of donor PGCs after transplantation if PGCs are labeled with a fluorescent marker.

3.2. Germ cell transplantation methods based on reproductive biology

Takeuchi and colleagues (2004) developed an innovative approach for the transplantation of PGCs that avoided the complexity of BT [54]. They isolated PGCs from the genital ridge of newly hatched trout embryos and transplanted the cells into the peritoneal cavities of host salmon embryos at the same stage as the donor (Fig. 1A). They termed this approach peritoneal cavity transplantation (PCT), and they obtained 13.5% female and 17% male normal germline chimeras that produced donor-derived gametes. Although the success rate of germline chimera production using this method was not as high as that previously reported for BT, BdT, and SPT, PGC transplantation into hatched larvae has considerable advantages, such as the high developmental rate after manipulation and the use of comparatively simple equipment and procedures, particularly in salmonid species that have larvae that are large enough for this type of manipulation. Subsequently, it was shown that spermatogonia (probably containing spermatogonial stem cells) and oogonia (probably containing oogonial stem cells) could be used as the donor cell sources for this technique [36,40]. The significance of using gonial cells isolated from adult fish is the simplicity of obtaining these cells from adult gonads and the ease of use for various cell operations, such as cryopreservation and cell culture. Surprisingly, a transplantable quantity and quality of gonial cells can be isolated from frozen testes [55,56], frozen ovary [57], and even whole frozen fish in a freezer [58]. Currently, spermatogonial or oogonial transplantation by PCT is considered the optimal strategy for the production of germline chimeras between different fish species (Fig. 1). However,

the application of PCT can be technically very difficult for species with small and fragile larvae, such as marine fish species. Tuna have small larvae that float and that do not have a large space in the peritoneal cavity to accept donor cells. The survival rate from hatchling to larvae in marine species is generally low; the additional mortality induced by experimental manipulation means that for some species, the number of chimeras produced by PCT will be insufficient for practical surrogate production.

To overcome the problems posed by small larvae, Franca and colleagues developed a new technique in which donor germline cells (that may include stem cells) are transplanted into the gonads of an adult fish via the genital pore. Genital pore transplantation (GPT) is a variation of the method for spermatogonial transplantation into seminiferous tubules that was established in mammals by Brinster and collaborators in the 1990s. Lacerda et al. (2006) isolated germ cells from testes of tilapia and labelled the cells with the fluorescent dye PKH26. Recipient adult fish were treated with busulfan and high temperatures to induce degeneration of endogenous germ cells prior to transplantation. The isolated tilapia germ cells were then transplanted into the spermatic ducts of the testis of the recipient via the genital papillae. Analysis of the recipient testes confirmed proliferation of the transplanted germ cells. In a subsequent study, they performed GPT with spermatogonia from other tilapia species and obtained donor-derived offspring (6.3%; 2 out of 32) using semen from germline chimeras [53]. Majhi et al. (2009) applied the same technique to pejerrey species (genus *Odontesthes*) and obtained donor-derived sperm with 1.2%–13.3% germline transmission [52]. Majhi et al. (2014) also demonstrated that donor oogonia isolated from *O. bonariensis* and transplanted by GPT could proliferate in the ovary of *O. hatcheri*; a progeny test showed that xenogeneic eggs were produced in the germline chimeras [59]. GPT has also been used in zebrafish [50]. The most significant advantage of this technique is the low level of physical damage to the recipient; additionally, the technique requires only simple equipment, such as a syringe. GPT can be conducted easily without the need for intensive practice in the use of micromanipulation devices, as in the case of other transplantation methods. Donor-derived gametes (especially sperm) can be obtained in a comparatively short period when compared to other methods—especially those in which transplantation is performed in the embryonic or early developmental stages. The latter method requires that the chimera are raised until adulthood so that gametes can be obtained. However, GPT requires a large number of isolated donor cells before transplantation, as adult host fish have large spaces in their gonads. In tilapia model, 10^7 isolated gonial cells in 1 ml were injected into a single fish [53], whereas 10^4 gonial cells were enough for

inducing germline chimera in PCT in trout [60]. The transplantation is also frequently combined with a reduction in the number of endogenous germ cells, for example, through the use of chemicals, such as busulfan, and the production of a completely sterile host is difficult.

4. Development of PGCs in fish

4.1. Study of PGC for surrogate production

As described above, spermatogonial and oogonial cells have been used as donor sources for PCT and GPT to produce germline chimeras. The crucial steps in germ cell transplantation, such as techniques to sterilize hosts, select host stages for transplantation, and visualize germ cells, are based on information on PGC specification, proliferation, and migration. In fish, PGCs differentiate outside of the embryonic gonadal region and migrate toward the gonadal ridge where they proliferate and associate with somatic cells to form the gonads. The details of these processes are described below.

4.2. Specification of fish PGCs by germplasm

Information on PGC specification, survival, and migration is essential to the development of techniques for PGC manipulation. Molecular tools and transgenic strains have been developed to study PGC specification and migration in detail during embryonic development, especially in model fish species. PGCs are specified by the inheritance of maternally supplied cytoplasm determinants, termed germ plasm, during the cleavage period and are the first cell type specified from undifferentiated embryonic cells in the embryo. At the early blastula stage, PGCs that contain germ plasm are specified in the peripheral region of the lower blastoderm in cyprinids and the central region of the lower blastoderm in some marine species [61,62]. In zebrafish, the germ plasm is located at both ends of the first and second cleavage furrows, and four PGCs eventually differentiate in each of the peripheral regions that originate from the furrows of blastula embryos [25]. Germ plasm is a complex collection of mitochondria, fibrils, and specific mRNAs and proteins that act as translational regulators; it accumulates in the Balbiani body at the vegetal hemisphere of developing oocytes, although some germline-specific proteins, such as VASA, do not localize in this aggregation [63]. In zebrafish, it has been shown that the *bucky ball* (*buc)* gene has a role as a germ

plasm organizer in the Balbiani body during oogenesis and early embryonic development. In the absence of *buc* expression, the germ plasm fails to aggregate during the embryonic stage, and oocytes lose polarity during oogenesis [64,65]. To date, Buc protein has only been identified in vertebrates; in insects, Osker (Osk) protein has a function similar to that of Buc [66] [67]. Interestingly, although Buc and Osk do not possess homologous protein motifs, Osk functions identically to Buc when injected into zebrafish eggs [68]. Injection of *buc-GFP* mRNA into one-cell-stage fish embryos results in the visualization of the germ plasm in embryos by the Buc-GFP fusion protein in the marginal region of the first two cleavage furrows; however, the visualized germ plasm loses the GFP tag after the blastula stage [65]. Blastomeres that inherit these germ plasm aggregations at the cleavage furrow are specified as PGCs while cleavage proceeds. The function of *buc* is widely conserved among fish species. A zebrafish Buc-GFP fusion protein was shown to localize and visualize the germ plasm in cleavage-stage embryos of sturgeon and salmon, indicating that PGCs in these fish are also specified by inheritance of the germ plasm. [33,66]. Overexpression of *buc* mRNA increases the number of PGCs during embryonic development in zebrafish, probably because the additional Buc protein recruits more germ plasm components in the blastoderm [65]. In contrast to that in zebrafish, the number of PGCs in medaka can be increased by overexpression of the dead end (*dnd*) gene in the same manner as that for *buc* overexpression in zebrafish [24,69]. The Buc-GFP fusion protein is expected to be of value in the transplantation of early-stage PGCs and the confirmation of PGC transfer into the host. Furthermore, boosting the number of PGCs by overexpression of a key gene related to germplasm formation is useful for inducing germline chimeras.

4.3. Methodologies to visualize PGCs in fish

In fish, PGC proliferation and migration have been studied in depth using the expression of germline marker genes, such as *vasa* and *nanos3* (*nos3*) [25,70-73]. The *vasa* gene encodes an RNA-binding protein with an ATP-dependent RNA helicase that is a member of the DEAD box family. Expression of *vasa* has been used as a germ cell marker throughout the whole process of gonadal development. The *nos3* gene is germ cell specific, and its 3'UTR contains a specific signal to a target and extends the expression in PGCs [74-76]. Transgenic fish strains that express GFP in germ cells driven by the vasa promoter have been used for studying PGC migration (rainbow trout [77], medaka [78], and zebrafish [79]). A GFP-*vasa* 3'UTR fusion construct under the *Xenopus* EF1α promoter has also been used to produce a transgenic line that expresses GFP exclusively in germ

cells [80]. In addition, injection of synthesized mRNA that combines GFP and *nos3* or *vasa* 3'UTR sequences into fertilized eggs can also be used to visualize embryonic PGCs in many species of fish [74,81,82]. *Nos3* 3'UTR-containing mRNAs are stabilized in PGCs but are subject to rapid degradation in somatic cells. Thus, *GFP-nos3* 3'UTR mRNA is expressed exclusively in PGCs during embryonic development. The function of *nos3* 3'UTR is widely conserved among fish species; thus, a construct produced from a zebrafish sequence can be used to visualize PGCs in fish species belonging to a wide range of taxonomic groups [32,33,83]. In sturgeon, PGCs can also be visualized by the injection of FITC-dextran (MW: 500,000) into the vegetal pole at early cleavage stages [84]. However, this technique has not yet been tested in other fish species.

4.4. Proliferation of PGCs

The number of PGCs does not increase during the first 4 hours of embryonic development until the 1,000-cell stage (1k) [25]. Asymmetric inheritance of the germ plasm, which is inherited by only one of the two dividing blastomeres, keeps the number of PGCs constant until the 1k stage [63]. At about the sphere stage, the germ plasm becomes more diffuse and spreads throughout the cell cytoplasm; the germ plasm is then symmetrically distributed between dividing blastomeres. PGCs increase in number gradually, as they migrate toward the genital ridge. The final number of PGCs in 24 hpf embryos varies among zebrafish strains: about 26 in the TL line and about 46 in the AB line [85]. The number of PGCs also varies among fish species: 8–11 in olive flounder (*Paralichthys olivaceus*) at the hatching stage (WISH with *nanos3* probe) [73], 53.5 in Atlantic salmon (*Salmo salar*) at the hatching stage (WISH with *vasa* probe) [86]; 21 in turbot (*Scophthalmus maximus*) at the 10-somite stage (WISH with vasa probe) [87], 5.6 in Japanese eel (*Anguilla japonica*) at the hatching stage (GFP-*nos3* 3'UTR mRNA injection) [32], and 42–64.5 (the variation in estimates between studies may be attributable to differences in the quality of the eggs as well as the genetic background of the egg batches) in sterlet sturgeon (*Acipenser ruthenus*) (FITC-dextran labeling). The available evidence indicates that the number of PGCs during the embryonic stage is less than 100 in a range of fish species.

4.5. Migration of PGCs from blastula to somitogenesis

In zebrafish, PGCs switch from a non-motile to a motile mode at the blastula stage and then begin to migrate [88]. PGCs at the early blastula stage (until 3.5 hpf) display a simple round shape similar to the surrounding somatic cells. Subsequently, they develop a non-polarized shape with complex protrusions (3.5–4.5 hpf) and then exhibit a polarized morphology (after 4.5 hpf) [88]. After 4.5 hpf, the PGCs respond to the guidance cues provided by chemokine signals and start migrating toward the genital ridge. The migrating PGCs show downregulation of E-cadherin expression compared to earlier developmental stages, suggesting that a reduction in cell–cell affinity between PGCs and somatic cells is important for the initiation of migration. Chemokine stromal-derived factor 1a (SDF1a) is secreted by somatic cells along the route of PGC migration, and its receptor CXCR4b is expressed on the surface of the PGCs; these signal and receptor molecules are key players in PGC migration. Migrating PGCs are guided toward the embryonic domain where the level of SDF1a is higher [82,89]. In zebrafish, a second SDF1 receptor, CXCR7, ensures accurate PGC migration. CXCR7 is expressed on the surface of somatic cells; it functions as a decoy receptor and rapidly scavenges excess SDF1a by internalization [89]. Thus, CXCR7 enables dynamic changes in the PGC attractant without affecting *sdf1a* transcription levels to ensure correct migration. Knockdown of CXCR7 results in the formation of ectopic PGCs [89]. The results of SPT experiments demonstrated that the molecular mechanisms controlling PGC migration are conserved among most fish species (SPT in Table 1). Interestingly, transplanted sturgeon PGCs can migrate to the genital ridge of goldfish despite differences in the migratory patterns of PGCs in each species [32]. It is likely that the different PGC migratory patterns in each fish species reflect differences in spatial size and temporal shifts in chemoattractant domains.

3.6. Migration of PGCs after somitogenesis

The final section of the PGC migratory pathway is through the mesoderm on the developing gut and the mesentery to the genital ridges (the dorsal part of the body cavity) after the somitogenesis period. In medaka and sturgeon, histological observations show that migrating PGCs on the developing gut are surrounded by mesodermal somatic cells. The PGCs then move toward the genital ridges together with the surrounding somatic cells [90,91]. The speed of PGC migration is drastically reduced at this stage compared with those of earlier stages in sturgeon [84]. This observation suggests that PGCs migrate in a coordinated manner with surrounding somatic cells on

the way through the mesoderm on the gut and the mesentery to the genital ridge. In *Xenopus*, the extracellular matrix protein fibronectin is essential for the correct migration of PGCs at the corresponding stage of development [92]. However, in Nibe croaker, PGCs are enclosed by somatic cells only after they reach the genital ridge [93]. In these species, the SDF1a/CXCR4 chemoattractant mechanism is assumed to be also involved in this PGC migratory stage, but this has not yet been clearly demonstrated. In the zebrafish *odysseus* mutant, in which the *cxcr4b* gene has a nonsense mutation, the disruption of PGC migration has been observed; approximately 46% of homozygous mutant embryos have small numbers of PGCs in the gonads, and a similar proportion of adult mutants is fertile [94]. This suggests that a small number of PGCs could migrate successfully to the genital ridge through the mesodermal tissue on the gut and the mesentery, although these cells are thought to lack SDF1a and CXCR4 chemoattractant signaling.

After migration, PGCs settle in the genital ridge where the gonads will be formed. Currently, the mechanism responsible for the termination of PGC migration in fish remains unknown; the duration of migratory ability in PGCs is possibly predetermined autonomously. When PGCs are isolated from different developmental stages and individually transplanted into blastula hosts, the migration rates of later stage PGCs toward the genital ridge are significantly lower than those of earlier stages [30]. Interestingly, when the migratory rate of a single transplanted PGC in a zebrafish host is compared among zebrafish, pearl danio, goldfish, and loach, the highest rate of migration is found for goldfish (about 60%) and not zebrafish (about 40%). This counterintuitive result may reflect differences in the *preloaded* migratory capacity of PGCs in each species to accommodate their own migratory pathways (Fig. 3). However, as described above, isolated PGCs from the genital ridges of newly hatched trout larvae can localize in the host genital ridges after transplantation into the peritoneal cavities [54]. Differences between the migratory behavior of PGCs before and after the somitogenesis period likely exist. Spermatogonia and oogonia transplanted into the peritoneal cavity migrate toward the genital ridge in the same manner as PGCs do after the somitogenesis period. To optimize and improve PCT for surrogate production, a better understanding of the final migratory step of PGCs to the genital ridge is vital.

5. Sex controls by germline chimera production

Control of the sex of seedlings is important for efficient production in aquaculture. Mono-sex populations are desirable if species show differences in growth between females and males, such as faster growth in the former in flatfish, or produce high-value gametes in either sex, such as sturgeon egg caviar and the testis of tiger puffer. In general, mono-sex populations can be produced by chromosomal manipulation and/or hormonal treatment of the parents [95]. The use of germline chimeras as spawning adults might be simpler and preferable for the control of seedling sex if a detailed understanding of the genotype and phenotype sex of germline chimeras and their gametes is available.

Fish possess a variety of sex-determining systems and sexual differentiation mechanisms [96]. Overall, genotypic/chromosomal sex determines physiological sex, which is what occurs in higher vertebrates. However, in some fish species, physiological sex can be altered by environmental factors, such as temperature, pH, and social cues. Gonadal development and sex differentiation processes also vary among species. Analyses of germline chimeras provide a valuable means for the investigation of sex determination and differentiation and for the characterization of gonadal development and sexual differentiation processes at the cellular level. PGC depletion during embryogenesis has been shown to cause masculinization or sex reversal from female to male in zebrafish and medaka but not in goldfish, loach, and Atlantic salmon [34,97-100]. Siegfried and Nusslein-Volhard (2008) reported that inhibiting *dnd* expression caused the complete depletion of PGCs before the cells reached the genital ridge and resulted in masculinization in zebrafish. This finding indicates that PGCs are required for ovarian differentiation in zebrafish. Tzung et al. (2015) investigated the role of PGCs in sex differentiation by replacement of the germ cells and transplantation of different numbers of PGCs by SPT and BdT [35]. Chimeras that possessed a single PGC became functional males, indicating that a single PGC was not sufficient for ovarian differentiation. However, 20% of the chimeras that had up to nine PGCs became female. This outcome was confirmed by injecting different doses of *dnd* morpholinos to produce morphants with a reduced number of PGCs. This study indicated that the number of PGCs is important for ovarian differentiation in zebrafish; however, the mechanisms of this phenomenon remain unknown. It was thought that PGC-dependent ovarian differentiation occurs in zebrafish because of its unclear chromosomal sex determination and undifferentiated gonochoristics. Goto et al. (2012) investigated goldfish sex differentiation as a representative of an XX/XY sex chromosome determination system [34]. They found that complete depletion of PGCs did not alter goldfish phenotypic sex and that SPT chimeras also developed as either female or male. These findings indicated that goldfish sex is determined by gonadal somatic cells or by chromosomal sex, regardless of whether any PGCs are

present. Similar findings have been reported for loach [99]. In contrast to the result in goldfish, medaka *cxcr4* morphants with complete depletion of PGCs before the initiation of gonadal morphogenesis show sex reversal in XX individuals [98]. However, some *cxcr4* morphants that had a small number of remaining PGCs became male; this suggests that the presence of a small number of PGCs before the formation of the gonad could masculinize XX individuals. The gonads of XX *cxcr4* morphants without any PGCs express genes that are required for ovarian differentiation at 10 dph, but this pattern of expression later disappears, and the genes for testicular differentiation are expressed at 20 dpf. These results indicate that ovarian supporting somatic cells could not maintain the expression of *foxl2* and *aromatase* if there are no germ cells and that they eventually transdifferentiate from ovarian supporting cells to testicular supporting cells. In danio species and salmonids, PCT germline chimeras produced using hybrids or triploids as hosts show the development of donor-germ cells into gametes in accordance with the host gonadal environment, regardless of whether the donor-germ cells were prepared from the testis or the ovary [36,41]. This indicates that the genotype and phenotype of some donor-derived gametes are inconsistent if the target species has a chromosomal sex-determining system. The inconsistency of sex between a donor and a host in germline chimeras can be advantageous for producing mono-sex populations for aquaculture, as shown in rainbow trout [101]. Okutsu et al. successfully demonstrated the production of Y eggs through female germline chimeras, which were transplanted with spermatogonial cells derived from XY male, and then generated YY *super-males* just by inseminating the Y eggs with milt from normal males. The production of Y eggs derived from transplanted spermatogonial cells has also been shown in tiger puffer, *T. rubrips* [48]. In this experiment, the spermatogonial cells of tiger puffer were transplanted into sterile triploids of the grass puffer, *T. niphobles*, and donor-derived eggs and sperm were obtained from the germline chimeras. The tiger puffer, *T. rubrips*, is a financially valuable species in terms of the restaurant industry in Japan. The price of a male is higher than that of a female, so mono-sex male production by using the milt of YY super-male of the tiger puffer is a great advantage for aquaculture. A detailed understanding of the sex determination/differentiation system and the pattern of gonadal development of target species enables us to utilize germline chimeras more efficiently for seedling production in practical aquaculture.

6. Sterilization of the host

The efficient and reliable production of donor-derived gametes through germline chimeras requires sterilization of the host. In fish, induced sterilization has been studied in aquaculture species since the early 1990s [95,102], and several approaches have been developed to induce sterility. The level of sterility and the physiological conditions of infertility differ between sexes and species. The contribution of donor-derived gametes is increased with host infertility, suggesting that competition might occur between donor and host germ cells during differentiation and proliferation in germline chimeras. Therefore, preparation of the host fish is a vital aspect of surrogate propagation. In xenogeneic transplantation, host sterilization is crucial to prevent unwanted hybrid offspring. Therefore, there was considerable motivation to develop secure and optimal methods; in general, these exploit the features of gonadal development and gametogenesis from egg to adult in each potential host species. Sterilization can be induced by chromosomal manipulation, gene knockdown or knockout, ultraviolet (UV) irradiation, and chemical treatment.

6.1. Chromosomal manipulation for the induction of triploids

The production of triploid fish by induced retention of the second polar body after normal fertilization has been achieved in many fish species [95,102]. In general, triploid fish are sterile, as the presence of three sets of chromosomes adversely affects meiosis [103,104]. Therefore, the ovaries of female triploids are considerably smaller than those of diploid fish and mainly contain oogonia, germ cells in early meiotic stages, and some peri-nucleolar and previtellogenic oocytes [102,105]. The gonads of male triploids develop to a similar size as those of diploids before maturation; however, primary spermatocytes develop abnormally. The gonads of both triploid females and males occasionally possess mature oocytes and sperm in some species; however, the cells are aneuploid, and any embryos produced in crosses with diploid fish do not develop normally. In salmonid species, xenogeneic transplantation of rainbow trout spermatogonia into triploid masu salmon hosts resulted in the production of 100% donor-derived offspring in a cross with surrogate parents [41]. By contrast, the same combination of donor and host species but using diploid fish (without any sterilization) as a host resulted in only 16.7% of germ cell transplanted hosts possessing donor-derived PGCs 1 month after transplantation, and the contribution of donor-derived sperm to F_1 offspring was low (0.4%) [54]. The use of triploids as a host is therefore a viable approach to surrogate propagation, but this type of chromosomal manipulation is only available for species in

which eggs and sperm can be obtained. Furthermore, in zebrafish, Delomas and Dabrowski (2018) reported that all triploids develop into males, suggesting that triploidy interferes with oocyte development and thereby induces male development, such as in zebrafish *fancl* mutants [106]. Understanding both genetic and phenotypic sex is also important for triploid hosts [107].

6.2. Hybridization

Hybrid sterility can also be used for the host and for xenogeneic transplantation [36,44,54]. Wong et al. (2011) produced hybrids between female zebrafish and male pearl danio and used 2-week-old hybrids for zebrafish oogonial transplantation. Yoshikawa et al. (2018) examined the sterility and viability of hybrids between inter- and intra-generic crosses for four sciaenid species. The intra-generic hybrids between *Nibea mitsukurii* and *N. albiflora*, which are genetically close, were fertile; however, an inter-generic cross between *N. mitsukurii* and *Pennahia argentata* was sterile, whereas the cross *N. mitsukurii* and *Argyrosomus japonicus* was inviable. Twelve dph sterile sciaenid hybrids were used as the host for the transplantation of spermatogonial cells from the species used to produce the hybrids. Donor-derived offspring were successfully obtained from male hybrids. Interestingly, danio hybrids developed into both female and male fish, but only male hybrids produced donor-derived gametes; female hybrids failed to develop zebrafish gametes, and their ovaries resembled those of non-transplanted female hybrids. In sciaenid hybrids, sterile hybrids were completely masculinized with mitotic arrest of the germ cells. Transplantation of spermatogonial cells in the sterile hybrids induced the recovery of the testis and the ovary (1/10) of the transplanted hybrids. Inviable hybrids are a valuable asset for preventing unwanted offspring produced from xenogeneic germline chimeras [54].

6.3. Inhibiting gene translation

Gene knockdown can be used to induce sterility when the target genes are essential for germ cell survival. *Dead end* (*dnd*) and *nos3*, first reported as *nanos1* in zebrafish, are the genes generally manipulated in fish. Maternally supplied *dnd* and *nos3* express in the cleavage furrows of early embryos and continue expression in PGCs [74,108,109]. These genes are essential for the

survival and migration of PGCs toward the genital ridge during embryogenesis. Inhibition of *dnd* expression by injecting morpholino oligos results in germ cell ablation in several species of fish, including zebrafish, medaka, goldfish, and sturgeon [24,34,97,100,110]. Similarly, inhibition of *nos* in zebrafish and medaka affects PGC migration [74,98]. For the complete depletion of PGCs before the initiation of gonadal morphogenesis, injecting morpholino oligos into eggs is necessary. This timing of injection in relation to embryo developmental stage is important, as is the concentration of morpholino and the use of an appropriate sequence against the target gene to avoid off-target effects and a low survival rate. In zebrafish, goldfish, and medaka, xenogeneic and allogeneic germline chimeras have been produced using sterilized hosts following the injection of morpholino oligos; in these chimeras, the transplanted cells completely replaced the host gametes [22,24,31,34]. Morpholino oligos can also be conjugated with a molecular transporter and are termed vivo-morpholino oligos; these modified oligos have been used to induce sterilization in zebrafish [111]. In zebrafish, immersion of in vivo fertilized eggs in vivo-morpholino oligos against *dnd* induced complete loss of PGCs; however, infertility was reduced when naturally spawned eggs were used. Higher concentrations of vivo-morpholino oligos cause malformations in embryos, so identifying the optimal dose of the compound that effectively induces sterility is important.

6.4. UV irradiation

In sturgeon, eliminating PGCs by UV irradiation of the vegetal pole where the germ plasm is localized during early cleavage stages is possible [112]; this method was first performed in frogs more than 50 years ago [113,114]. The developmental pattern of sturgeons is similar to that of amphibians: sturgeon eggs undergo holoblastic cleavage. Cell lineage analysis in sturgeon eggs shows that although the somatic cell lineage mainly originates from the animal hemisphere of the eggs, germ cells are derived from extraembryonic yolky blastomeres located at the vegetal pole [33]. Thus, UV irradiation of the vegetal pole does not affect embryonic development, except for PGCs.

6.5. Chemical treatment

Busulfan (myleran) is an alkylating agent that can interfere with cell division by cross-linking DNA strands. Treatment of hosts with busulfan was first carried out in mammals **[115]**. In fish, peritoneal injection of busulfan reduces endogenous germ cells in sexually competent male and female gonads in zebrafish, pejerrey, and tilapia **[50,53,116]**. In these species, busulfan treatment combined with a high temperature enhances germ cell degeneration for host preparation (called thermo-chemical treatment). This method cannot remove all germ cells in the gonad, as high doses of busulfan show toxicity not only to germ cells but also to other cells and cause severe side effects. Therefore, any remaining endogenous germ cells can recover and produce gametes in both the testis and the ovary. The advantage of busulfan treatment is that it induces germ cell degeneration through a simple peritoneal injection, therefore offering a relatively easy manipulation approach for host preparation. However, from an animal welfare perspective, the busulfan treatment needed to remove endogenous germ cells may effectively require the exposure of fish to the maximum tolerated dose, which might compromise their immune system.

6.6. Gene modification

Gene modification using TALEN and CRISPR/Cas9 technologies can be used to generate strains with the desired traits in any type of fish, including aquaculture species [117]. Mutagenesis of *nos2* and *nos3* by CRISPR/Cas9 in medaka induces PGC depletion and masculinization [117]. Similarly, knockout of *dnd* by CRISPR/Cas9 ablates PGCs in Atlantic salmon [100]. Knocking out the genes involved in germline development and fertility renders fish infertile. Therefore, maintaining such infertile strains is a challenge that requires a well-planned strategy. There have been few reports on inducible or controllable sterilization in zebrafish. Wong et al. (2013) generated a transgenic strain in which hsp70 regulates heat-shock inducible SDF1a expression in PGCs, and PGC migration was disrupted by the ubiquitous expression of SDF1a in PGCs by applying a high-temperature treatment on embryos [118]. In this way, sterilized fish are obtained, as needed. Using a Gal4/UAS system, Zhang et al. (2015) also developed a controllable, on–off reproductive containment strategy for zebrafish that renders the offspring sterile but leaves the parents fertile [119]. Recently, Nagasawa et al. (2018) established a system that combined surrogate technology and gene editing to produce a sterile mono-sex population that carried a mutated *follicle-stimulating receptor* (*fshr*) gene [120]. In this study, sex-reversed XX

spermatogonial cells (*fshr* (-/-)) were transplanted into sterile hybrids to produce *fshr* (-/-) eggs. These germline chimeras were fertile and crossed with sex-reversed XX (*fshr* (-/-)); they produced only infertile female offspring. These various reports show that gene modification technologies offer a powerful tool to generate optimal characteristics in potential hosts and thus can be used for inducing sterility. However, the role and usage of gene-modified fish for surrogate propagation need wider discussion and consideration, particularly its practical use. Inducible or controllable sterilization might be beneficial for aquaculture usage as a prerequisite trait for any type of induced mutant to prevent pollution of the gene pools of wild stock.

7. Future perspective for aquaculture

As described above, with regard to surrogate propagation, many different approaches can be applied to produce germline chimeras in fish. The choice of methodology depends on which is most appropriate for the target species involved. The chart in Figures 4 and 5 summarizes the methods for transplantation and sterilization and the species to which these can be applied. Research in surrogate propagation initially sought to develop xenogeneic germline chimeras in order to conserve endangered species. However, surrogate production has recently become more important for practical use in aquaculture, so methods for producing allogeneic germline chimeras have been developed for commercially important species. Surrogate production can accelerate the breeding of fish if transplantation is performed using cryopreserved germ cells from fish that possess desirable characteristics for farming, such as high growth and good survival after exposure to fish diseases or red-tide plankton. There are aspects of the various technologies available that need to be kept in mind. For example, in finfish culture in open water, preventing escapees from the net pan is difficult; therefore, hosts that have not been evaluated for safety with respect to the transmission of unwanted characters (such as gene modifications), hybrids, and inconsistent genotype/phenotype sex should not be used. The escape of xenogeneic germline chimeras or genome-modified fish into the wild might cause genetic introgression in the future. In the current situation of fish farming and in terms of social acceptability, allogeneic transplantation with sterilization of hosts by gene knockdown, chemical treatment, or officially approved triploids might be acceptable for finfish farming in floating net cages, such as for tuna. If germline chimeras are maintained in tanks away from open water and with secure management, then xenogeneic transplantation or hybrid sterility might be more acceptable for practical use. In the future, surrogate propagation will be a system for seedling

production in aquaculture. From the perspective of the practical use of this system, we need to examine the growth performance, health, and total quality of seedlings obtained using the technology. Additionally, the genetic management of target species is important for the conservation of endangered species.

Acknowledgement

We are deeply grateful to the members of the South Ehime Fisheries Research Center, Ehime University, the Faculty of Fisheries and Protection of Waters, the University of South Bohemia in Ceske Budejovice, the Nanae Fresh Water laboratory, the laboratory of Aquaculture Genetics and Genomics, and Hokkaido University, who patiently supported us with our research through the years. This study has been supported by JSPS KAKENHI grant numbers 16K07874 to TS and 17H03873/17K19294 to RG.

References

[1] Mudge K, Janick J, Scofield S, Goldschmidt EE. A History of Grafting. In: Janick J, editor. Horticultural Reviews, vol. 35, John Wiley & Sons, Inc; 2009, pp. 437–93. doi:10.1002/9780470593776.ch9.

[2] Spemann H, Schotté O. Über xenoplastische Transplantation als Mittel zur Analyse der embryonalen Induktion. Naturwissenschaften 1932;20:463–7. doi:10.1007/BF01504985.

[3] Le Douarin NM, Teillet MA. The migration of neural crest cells to the wall of the digestive tract in avian embryo. J Embryol Exp Morphol 1973;30:31–48.

[4] Technau GM, Campos-Ortega JA. Lineage analysis of transplanted individual cells in embryos of *Drosophila melanogaster* : III. Commitment and proliferative capabilities of pole cells and midgut progenitors. Rouxs Arch Dev Biol 1986;195:489–98. doi:10.1007/BF00375889.

[5] Blackler AW. Transfer of primordial germ-cells between two subspecies of *Xenopus laevis*. J Embryol Exp Morphol 1962;10:641–51.

[6] Ikenishi K, Tanaka TS. *Xenopus Heterochronic* Presumptive Primordial Germ Cells (pPGCs) Implanted in the Correct Position in Host Neurula Embryos can Differentiate into PGCs. Dev Growth Differ 1993;35:439–45. doi:10.1111/j.1440-169X.1993.00439.x.

[7] Fehilly CB, Willadsen SM, Tucker EM. Interspecific chimaerism between sheep and goat. Nature 1984;307:634–6. doi:10.1038/307634a0.

[8] Ogawa T, Dobrinski I, Avarbock MR, Brinster RL. Xenogeneic spermatogenesis following transplantation of hamster germ cells to mouse testes. Biology of Reproduction 1999;60:515–21.

[9] França LR, Ogawa T, Avarbock MR, Brinster RL, Russell LD. Germ cell genotype controls cell cycle during spermatogenesis in the rat. Biology of Reproduction 1998;59:1371–7.

[10] Clouthier DE, Avarbock MR, Maika SD, Hammer RE, Brinster RL. Rat spermatogenesis in mouse testis. Nature 1996;381:418–21.

[11] Honaramooz A, Megee SO, Dobrinski I. Germ Cell Transplantation in Pigs. Biology of Reproduction 2002;66:21–8. doi:10.1095/biolreprod66.1.21.

[12] Honaramooz A, Behboodi E, Blash S, Megee SO, Dobrinski I. Germ cell transplantation in goats. Mol Reprod Dev 2003;64:422–8. doi:10.1002/mrd.10205.

[13] Hermann BP, Sukhwani M, Winkler F, Pascarella JN, Peters KA, Sheng Y, et al. Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. Cell Stem Cell 2012;11:715–26. doi:10.1016/j.stem.2012.07.017.

[14] Eppig JJ, Wigglesworth K. Development of mouse and rat oocytes in chimeric reaggregated ovaries after interspecific exchange of somatic and germ cell components. Biology of Reproduction 2000;63:1014–23.

[15] Zhao DF, Kuwana T. Purification of avian circulating primordial germ cells by Nycodenz density gradient centrifugation. British Poultry Science 2003;44:30–5. doi:10.1080/0007166031000085382.

[16] Nakajima Y, Minematsu T, Naito M, Tajima A. A New Method for Isolating Viable Gonadal Germ Cells from 7-day-old Chick Embryos. Jpn Poult Sci 2011;48:106–11. doi:10.2141/jpsa.010094.

[17] Nakamura Y. Avian Biotechnology. Adv Exp Med Biol 2017;1001:187–214. doi:10.1007/978-981-10-3975-1_12.

[18] Wakamatsu Y, Ozato K, Hashimoto H, Kinoshita M, Sakaguchi M, Iwamatsu T, et al.

Generation of germ-line chimeras in medaka (*Oryzias latipes*). Mol Mar Biol Biotech 1993;2:325–332–332.

[19] Takeuchi Y, Yoshizaki G, Takeuchi T. Production of germ-line chimeras in rainbow trout by blastomere transplantation. Mol Reprod Dev 2001;59:380–9. doi:10.1002/mrd.1044.

[20] Nakagawa M, Kobayashi T, Ueno K. Production of germline chimera in loach (*Misgurnus anguillicaudatus*) and proposal of new method for preservation of endangered fish species. J Exp Zool 2002;293:624–31. doi:10.1002/jez.10184.

[21] Güralp H, Pocherniaieva K, Blecha M, Policar T, Psenicka M, Saito T. Migration of primordial germ cells during late embryogenesis of pikeperch *Sander lucioperca* relative to blastomere transplantation. Czech Journal of Animal Science 2017;62:121–9. doi:10.17221/40/2016-CJAS.

[22] Ciruna B, Weidinger G, Knaut H, Thisse B, Thisse C, Raz E, et al. Production of maternal-zygotic mutant zebrafish by germ-line replacement. Proc Natl Acad Sci USa 2002;99:14919–24. doi:10.1073/pnas.222459999.

[23] Shimada A, Takeda H. Production of a maternal-zygotic medaka mutant using hybrid sterility. Dev Growth Differ 2008;50:421–6. doi:10.1111/j.1440-169X.2008.01051.x.

[24] Li M, Hong N, Xu H, Song J, Hong Y. Germline replacement by blastula cell transplantation in the fish medaka. Sci Rep 2016;6:29658. doi:10.1038/srep29658.

[25] Yoon C, Kawakami K, Hopkins N. Zebrafish vasa homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. Development 1997;124:3157–65.

[26] Yamaha E, Kazama-Wakabayashi M, Otani S, Fujimoto T, Arai K. Germ-line chimera by lower-part blastoderm transplantation between diploid goldfish and triploid crucian carp. Genetica 2001;111:227–36.

[27] Mizuno T, Yamaha E, Wakahara M, Kuroiwa A, Takeda H. Mesoderm induction in zebrafish. Nature 1996;383:131–2.

[28] Yamaha E, Mizuno T, Hasebe Y, Takeda H, Yamazaki F. Dorsal specification in blastoderm at the blastula stage in the goldfish, *Carassius auratus*. Dev Growth Differ 1998;40:267–75.

[29] Yamaha E, Murakami M, Hada K, Otani S, Fujimoto T, Tanaka M, et al. Recovery of fertility in male hybrids of a cross between goldfish and common carp by transplantation of PGC (primordial germ cell)-containing graft. Genetica 2003;119:121–31.

[30] Saito T, Goto R, Fujimoto T, Kawakami Y, Arai K, Yamaha E. Inter-species transplantation and migration of primordial germ cells in cyprinid fish. Int J Dev Biol 2010;54:1479–84. doi:10.1387/ijdb.103111ts.

[31] Saito T, Goto R, Arai K, Yamaha E. Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation. Biology of Reproduction 2008;78:159–66. doi:10.1095/biolreprod.107.060038.

[32] Saito T, Goto R, Kawakami Y, Nomura K, Tanaka H, Adachi S, et al. The mechanism for primordial germ-cell migration is conserved between Japanese eel and zebrafish. PLoS ONE 2011;6:e24460–. doi:10.1371/journal.pone.0024460.

[33] Saito T, Pšenička M, Goto R, Adachi S, Inoue K, Arai K, et al. The origin and migration of primordial germ cells in sturgeons. PLoS ONE 2014;9:e86861. doi:10.1371/journal.pone.0086861.

[34] Goto R, Saito T, Takeda T, Fujimoto T, Takagi M, Arai K, et al. Germ cells are not the primary factor for sexual fate determination in goldfish. Developmental Biology 2012;370:98–109.

[35] Tzung K-W, Goto R, Saju JM, Sreenivasan R, Saito T, Arai K, et al. Early depletion of primordial germ cells in zebrafish promotes testis formation. Stem Cell Reports 2015;4:61–73. doi:10.1016/j.stemcr.2014.10.011.

[36] Wong T-T, Saito T, Crodian J, Collodi P. Zebrafish Germline Chimeras Produced by Transplantation of Ovarian Germ Cells into Sterile Host Larvae. Biology of Reproduction 2011;84:1190–7. doi:10.1095/biolreprod.110.088427.

[37] Franěk R, Marinović Z, Lujić J, Urbányi B, Fučíková M, Kaspar V, et al. Cryopreservation and transplantation of common carp spermatogonia. bioRxiv 2018:429449. doi:10.1101/429449.

[38] Franěk R, Tichopád T, Steinbach C, Xie X, Lujić J, Marinović Z, et al. Preservation of female genetic resources of common carp through oogonial stem cell manipulation. Cryobiology 2019. doi:10.1016/j.cryobiol.2019.01.016.

[39] Seki S, Kusano K, Lee S, Iwasaki Y, Yagisawa M, Ishida M, et al. Production of the medaka derived from vitrified whole testes by germ cell transplantation. Sci Rep 2017;7:43185. doi:10.1038/srep43185.

[40] Okutsu T, Yano A, Nagasawa K, Shikina S, Kobayashi T, Takeuchi Y, et al. Manipulation of fish germ cell: visualization, cryopreservation and transplantation. J Reprod Dev 2006;52:685–93.

[41] Okutsu T, Shikina S, Kanno M, Takeuchi Y, Yoshizaki G. Production of trout offspring from triploid salmon parents. Science 2007;317:1517. doi:10.1126/science.1145626.

[42] Lujić J, Marinović Z, Bajec SS, Djurdjevič I, Urbányi B, Horváth Á. Interspecific germ cell transplantation: a new light in the conservation of valuable Balkan trout genetic resources? Fish Physiol Biochem 2018;44:1487–98. doi:10.1007/s10695-018-0510-4.

[43] Octavera A, Yoshizaki G. Production of donor-derived offspring by allogeneic transplantation of spermatogonia in Chinese rosy bitterling. Biology of Reproduction 2018;35:86. doi:10.1093/biolre/ioy236.

[44] Yoshikawa H, Xu D, Ino Y, Yoshino T, Hayashida T, Wang J, et al. Hybrid Sterility in Fish Caused by Mitotic Arrest of Primordial Germ Cells. Genetics 2018;209:507–21. doi:10.1534/genetics.118.300777.

[45] Morita T, Kumakura N, Morishima K, Mitsuboshi T, Ishida M, Hara T, et al. Production of Donor-Derived Offspring by Allogeneic Transplantation of Spermatogonia in the Yellowtail (*Seriola quinqueradiata*) 2012. Biology of Reproduction 2012;86:176. doi: 10.1095/biolreprod.111.097873.

[46] Pšenička M, Saito T, Linhartová Z, Gazo I. Isolation and transplantation of sturgeon early-stage germ cells. Theriogenology 2015;83:1085–92. doi:10.1016/j.theriogenology.2014.12.010.

[47] Ye H, Li C-J, Yue H-M, Du H, Yang X-G, Yoshino T, et al. Establishment of intraperitoneal germ cell transplantation for critically endangered Chinese sturgeon *Acipenser sinensis*. Theriogenology 2017;94:37–47. doi:10.1016/j.theriogenology.2017.02.009.

[48] Hamasaki M, Takeuchi Y, Yazawa R, Yoshikawa S, Kadomura K, Yamada T, et al. Production of Tiger Puffer *Takifugu rubripes* Offspring from Triploid Grass Puffer *Takifugu niphobles* Parents. Mar Biotechnol 2017;19:579–91. doi:10.1007/s10126-017-9777-1.

[49] Yazawa R, Takeuchi Y, Higuchi K, Yatabe T, Kabeya N, Yoshizaki G. Chub mackerel gonads support colonization, survival, and proliferation of intraperitoneally transplanted xenogenic germ cells. Biology of Reproduction 2010;82:896–904. doi:10.1095/biolreprod.109.081281.

[50] Nobrega RH, Greebe CD, van de Kant H, Bogerd J, França LR, Schulz RW. Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. PLoS ONE 2010;5. doi:10.1371/journal.pone.0012808.

[51] Silva MA, Costa GMJ, Lacerda SMSN, Brandão-Dias PFP, Kalapothakis E, Silva Júnior AF, et al. Successful xenogeneic germ cell transplantation from Jundia catfish (*Rhamdia quelen*) into adult Nile tilapia (*Oreochromis niloticus*) testes. General and Comparative Endocrinology 2016;230-231:48–56. doi:10.1016/j.ygcen.2016.03.012.

[52] Majhi SK, Hattori RS, Yokota M, Watanabe S, Strüssmann CA. Germ Cell Transplantation Using Sexually Competent Fish: An Approach for Rapid Propagation of Endangered and Valuable Germlines. PLoS ONE 2009;4:e6132. doi:10.1371/journal.pone.0006132.t001.

[53] Lacerda SMSN, Batlouni SR, Costa GMJ, Segatelli TM, Quirino BR, Queiroz BM, et al. A new and fast technique to generate offspring after germ cells transplantation in adult fish: the Nile tilapia (*Oreochromis niloticus*) model. PLoS ONE 2010;5:e10740. doi:10.1371/journal.pone.0010740.

[54] Takeuchi Y, Yoshizaki G, Takeuchi T. Biotechnology: surrogate broodstock produces salmonids. Nature 2004;430:629–30. doi:10.1038/430629a.

[55] Lee S, Iwasaki Y, Shikina S, Yoshizaki G. Generation of functional eggs and sperm from cryopreserved whole testes. PNAS 2013;110:1640–5. doi:10.1073/pnas.1218468110.

[56] Pšenička M, Saito T, Rodina M, Dzyuba B. Cryopreservation of early stage Siberian sturgeon *Acipenser baerii* germ cells, comparison of whole tissue and dissociated cells. Cryobiology 2016;72:119–22. doi:10.1016/j.cryobiol.2016.02.005.

[57] Lee S, Katayama N, Yoshizaki G. Generation of juvenile rainbow trout derived from cryopreserved whole ovaries by intraperitoneal transplantation of ovarian germ cells. Biochemical and Biophysical Research Communications 2016;478:1478–83. doi:10.1016/j.bbrc.2016.08.156.

[58] Lee S, Seki S, Katayama N, Yoshizaki G. Production of viable trout offspring derived from frozen whole fish. Sci Rep 2015;5:16045. doi:10.1038/srep16045.

[59] Majhi SK, Hattori RS, Rahman SM, Strüssmann CA. Surrogate Production of Eggs and Sperm by Intrapapillary Transplantation of Germ Cells in Cytoablated Adult Fish. PLoS ONE 2014.

[60] 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. PNAS 2006;103:2725–9. doi:10.1073/pnas.0509218103.

[61] Kitauchi T, Saito T, Motomura T, Arai K, Yamaha E. Distribution and function of germ plasm in cytoplasmic fragments from centrifuged eggs of the goldfish, *Carassius*

auratus. Journal of Applied Ichthyology 2012;28:998–1005. doi:10.1111/jai.12068.

[62] Goto R, Saito T, Kawakami Y, Kitauchi T, Takagi M, Todo T, et al. Visualization of primordial germ cells in the fertilized pelagic eggs of the barfin flounder *Verasper moseri*. Int J Dev Biol 2015;59:465–70. doi:10.1387/ijdb.150008rg.

[63] Knaut H, Pelegri F, Bohmann K, Schwarz H, Nüsslein-Volhard C. Zebrafish vasa RNA but not its protein is a component of the germ plasm and segregates asymmetrically before germline specification. The Journal of Cell Biology 2000;149:875–88.

[64] Bontems F, Stein A, Marlow F, Lyautey J, Gupta T, Mullins MC, et al. Bucky Ball Organizes Germ Plasm Assembly in Zebrafish. Current Biology 2009;19:414–22. doi:10.1016/j.cub.2009.01.038.

[65] Marlow FL, Mullins MC. Bucky ball functions in Balbiani body assembly and animal-vegetal polarity in the oocyte and follicle cell layer in zebrafish. Developmental Biology 2008;321:40–50. doi:10.1016/j.ydbio.2008.05.557.

[66] Škugor A, Tveiten H, Johnsen H, Andersen Ø. Multiplicity of Buc copies in Atlantic salmon contrasts with loss of the germ cell determinant in primates, rodents and axolotl. BMC Evol Biol 2016;16:232. doi:10.1186/s12862-016-0809-7.

[67] Quan H, Lynch JA. The evolution of insect germline specification strategies. Curr Opin Insect Sci 2016;13:99–105. doi:10.1016/j.cois.2016.02.013.

[68] Krishnakumar P, Riemer S, Perera R, Lingner T, Goloborodko A, Khalifa H, et al. Functional equivalence of germ plasm organizers. PLoS Genet 2018;14:e1007696. doi:10.1371/journal.pgen.1007696.

[69] Hong N, Li M, Yuan Y, Wang T, Yi M, Xu H, et al. Dnd Is a Critical Specifier of Primordial Germ Cells in the Medaka Fish. Stem Cell Reports 2016;6:411–21. doi:10.1016/j.stemcr.2016.01.002.

[70] Braat AK, Zandbergen T, van de Water S, Goos HJ, Zivkovic D. Characterization of zebrafish primordial germ cells: Morphology and early distribution of vasa RNA. Dev Dyn 1999;216:153–67.

[71] Shinomiya A, Tanaka M, Kobayashi T, Nagahama Y, Hamaguchi S. The vasa-like gene, olvas, identifies the migration path of primordial germ cells during embryonic body formation stage in the medaka, *Oryzias latipes*. Dev Growth Differ 2000;42:317–26.

[72] Presslauer C, Nagasawa K, Fernandes JM, Babiak I. Expression of vasa and nanos3 during primordial germ cell formation and migration in Atlantic cod (*Gadus morhua L.*). Theriogenology 2012;78:1262–77. doi:10.1016/j.theriogenology.2012.05.022.

[73] Li M, Tan X, Jiao S, Wang Q, Wu Z, You F, et al. A new pattern of primordial germ cell migration in olive flounder (*Paralichthys olivaceus*) identified using nanos3. Development Genes and Evolution 2015;225:195–206. doi:10.1007/s00427-015-0503-6.

[74] Köprunner M, Thisse C, Thisse B, Raz E. A zebrafish nanos-related gene is essential for the development of primordial germ cells. Gene Dev 2001;15:2877–85.

[75] Mishima Y, Giraldez AJ, Takeda Y, Fujiwara T, Sakamoto H, Schier AF, et al. Differential Regulation of Germline mRNAs in Soma and Germ Cells by Zebrafish miR-430. Current Biology 2006;16:2135–42. doi:10.1016/j.cub.2006.08.086.

[76] Kedde M, Strasser MJ, Boldajipour B, Oude Vrielink JAF, Slanchev K, le Sage C, et al. RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. Cell 2007;131:1273–86. doi:10.1016/j.cell.2007.11.034.

[77] 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;44:323–6.

[78] Tanaka M, Kinoshita M, Kobayashi D, Nagahama Y. Establishment of medaka (*Oryzias latipes*) transgenic lines with the expression of green fluorescent protein fluorescence exclusively in germ cells: a useful model to monitor germ cells in a live vertebrate. PNAS 2001;98:2544–9. doi:10.1073/pnas.041315498.

[79] Krøvel AV, Olsen LC. Expression of a vas::EGFP transgene in primordial germ cells of the zebrafish. Mechanisms of Development 2002;116:141–50.

[80] Knaut H, Steinbeisser H, Schwarz H, Nüsslein-Volhard C. An evolutionary conserved region in the vasa 3 ' UTR targets RNA translation to the germ cells in the zebrafish. Current Biology 2002;12:454–66.

[81] Yoshizaki G. Green Fluorescent Protein Labeling of Primordial Germ Cells Using a Nontransgenic Method and Its Application for Germ Cell Transplantation in Salmonidae. Biology of Reproduction 2005;73:88–93. doi:10.1095/biolreprod.104.034249.

[82] Kurokawa H, Aoki Y, Nakamura S, Ebe Y, Kobayashi D, Tanaka M. Time-lapse analysis reveals different modes of primordial germ cell migration in the medaka Oryzias latipes. Dev Growth Differ 2006;48:209–21. doi:10.1111/j.1440-169X.2006.00858.x.

[83] Saito T, Fujimoto T, Maegawa S, Inoue K, Tanaka M, Arai K, et al. Visualization of primordial germ cells in vivo using GFP-nos1 3'UTR mRNA. Int J Dev Biol 2006;50:691–9. doi:10.1387/ijdb.062143ts.

[84] Saito T, Pšenička M. Novel Technique for Visualizing Primordial Germ Cells in

Sturgeons (*Acipenser ruthenus, A. gueldenstaedtii, A. baerii*, and *Huso huso*). Biology of Reproduction 2015;93:biolreprod.115.128314. doi:10.1095/biolreprod.115.128314.

[85] Weidinger G, Wolke U, Köprunner M, Klinger M, Raz E. Identification of tissues and patterning events required for distinct steps in early migration of zebrafish primordial germ cells. Development 1999;126:5295–307.

[86] Nagasawa K, Fernandes JM, Yoshizaki G, Miwa M, Babiak I. Identification and migration of primordial germ cells in Atlantic salmon, *Salmo salar*: characterization of vasa, dead end, and lymphocyte antigen 75 genes. Mol Reprod Dev 2013;80:118–31. doi:10.1002/mrd.22142.

[87] Lin F, Xu S, Ma D, Xiao Z, Zhao C, Xiao Y, et al. Germ line specific expression of a vasa homologue gene in turbot (*Scophthalmus maximus*): Evidence for vasa localization at cleavage furrows in euteleostei. Mol Reprod Dev 2012;79:803–13. doi:10.1002/mrd.22120.

[88] Blaser H, Eisenbeiss S, Neumann M, Reichman-Fried M, Thisse B, Thisse C, et al. Transition from non-motile behaviour to directed migration during early PGC development in zebrafish. Journal of Cell Science 2005;118:4027–38. doi:10.1242/jcs.02522.

[89] Doitsidou M, Reichman-Fried M, Stebler J, Köprunner M, Dorries J, Meyer D, et al. Guidance of primordial germ cell migration by the chemokine SDF-1. Cell 2002;111:647–59.

[90] Grandi G, Chicca M. Histological and ultrastructural investigation of early gonad development and sex differentiation in Adriatic sturgeon (*Acipenser naccarii*, *Acipenseriformes*, *Chondrostei*). J Morphol 2008;269:1238–62. doi:10.1002/jmor.10657.

[91] Hamaguchi S. A light- and electron-microscopic study on the migration of primordial germ cells in the teleost, *Oryzias latipes*. Cell Tissue Res 1982;227:139–51.

[92] Heasman J, Hynes RO, Swan AP, Thomas V, Wylie CC. Primordial germ cells of Xenopus embryos: the role of fibronectin in their adhesion during migration. Cell 1981;27:437–47.

[93] Takeuchi Y, Higuchi K, Yatabe T, Miwa M, Yoshizaki G. Development of spermatogonial cell transplantation in Nibe croaker, *Nibea mitsukurii* (Perciformes, Sciaenidae). Biology of Reproduction 2009;81:1055–63. doi:10.1095/biolreprod.109.077701.

[94] Knaut H, Werz C, Geisler R, Nüsslein-Volhard C, Tübingen 2000 Screen Consortium. A

zebrafish homologue of the chemokine receptor Cxcr4 is a germ-cell guidance receptor. Nature 2003;421:279–82. doi:10.1038/nature01338.

[95] Arai K. Genetic improvement of aquaculture finfish species by chromosome manipulation techniques in Japan. Aquaculture 2001;197:205-228.

[96] Devlin RH, Nagahama Y. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 2002;208:191–364. doi:10.1016/S0044-8486(02)00057-1.

[97] Siegfried KR, Nüsslein-Volhard C. Germ line control of female sex determination in zebrafish. Developmental Biology 2008;324:277–87. doi:10.1016/j.ydbio.2008.09.025.

[98] Kurokawa H, Saito D, Nakamura S, Katoh-Fukui Y, Ohta K, Baba T, et al. Germ cells are essential for sexual dimorphism in the medaka gonad. PNAS 2007;104:16958–63. doi:10.1073/pnas.0609932104.

[99] Fujimoto T, Nishimura T, Goto R, Kawakami Y, Yamaha E, Arai K. Sexual dimorphism of gonadal structure and gene expression in germ cell-deficient loach, a teleost fish. PNAS 2010;107:17211–6. doi:10.1073/pnas.1007032107.

[100] Wargelius A, Leininger S, Skaftnesmo KO, Kleppe L, Andersson E, Taranger GL, et al. Dnd knockout ablates germ cells and demonstrates germ cell independent sex differentiation in Atlantic salmon. Sci Rep 2016;6:21284. doi:10.1038/srep21284.

[101] Okutsu T, Shikina S, Sakamoto T, Mochizuki M, Yoshizaki G. Successful production of functional Y eggs derived from spermatogonia transplanted into female recipients and subsequent production of YY supermales in rainbow trout, *Oncorhynchus mykiss*. Aquaculture 2015;446:298–302. doi:10.1016/j.aquaculture.2015.05.020.

[102] Piferrer F, Beaumont A, Falguière J-C, Flajshans M, Haffray P, Colombo L. Polyploid fish and shellfish: Production, biology and applications to aquaculture for performance improvement and genetic containment. Aquaculture 2009;293:125–56. doi:10.1016/j.aquaculture.2009.04.036.

[103] Cuñado N, Terrones J, Sánchez L, Martínez P, Santos JL. Sex-dependent synaptic behaviour in triploid turbot, *Scophthalmus maximus* (Pisces, Scophthalmidae). Heredity 2002;89:460–4. doi:10.1038/sj.hdy.6800165.

[104] Oliveira C, Foresti F, Rigolino MG, Tabata YA. Synaptonemal Complex Formation in Spermatocytes of the Autotriploid Rainbow Trout, *Oncorhynchus Mykiss* (Pisces, Salmonidae). Hereditas 1995;123:215–20. doi:10.1111/j.1601-5223.1995.00215.x.

[105] Takeuchi Y, Yatabe T, Yoshikawa H, Ino Y, Kabeya N, Yazawa R, et al. Production of

functionally sterile triploid Nibe croaker *Nibea mitsukurii* induced by cold-shock treatment with special emphasis on triploid aptitude as surrogate broodstock. Aquaculture 2018;494:45–56. doi:10.1016/j.aquaculture.2016.05.030.

[106] Delomas TA, Dabrowski K. Why are triploid zebrafish all male? Mol Reprod Dev 2018;85:612–21. doi:10.1002/mrd.22998.

[107] Rodríguez-Marí A, Cañestro C, Bremiller RA, Nguyen-Johnson A, Asakawa K, Kawakami K, et al. Sex reversal in zebrafish fancl mutants is caused by Tp53-mediated germ cell apoptosis. PLoS Genet 2010;6:e1001034. doi:10.1371/journal.pgen.1001034.

[108] Weidinger G, Stebler J, Slanchev K, Dumstrei K, Wise C, Lovell-Badge R, et al. dead end, a Novel Vertebrate Germ Plasm Component, Is Required for Zebrafish Primordial Germ Cell Migration and Survival. Current Biology 2003;13:1429–34. doi:10.1016/S0960-9822(03)00537-2.

[109] Wang X, Liu Q, Xiao Y, Yang Y, Wang Y, Song Z, et al. The dnd RNA Identifies Germ Cell Origin and Migration in Olive Flounder (*Paralichthys olivaceus*). Biomed Res Int 2015;2015:428591. doi:10.1155/2015/428591.

[110] Linhartová Z, Saito T, Kaspar V, Rodina M, Prášková E, Hagihara S, et al. Sterilization of sterlet *Acipenser ruthenus* by using knockdown agent, antisense morpholino oligonucleotide, against dead end gene. Theriogenology 2015;84:1246–1255.e1. doi:10.1016/j.theriogenology.2015.07.003.

[111] Wong T-T, Zohar Y. Production of reproductively sterile fish: A mini-review of germ cell elimination technologies. General and Comparative Endocrinology 2015;221:3–8. doi:10.1016/j.ygcen.2014.12.012.

[112] Saito T, Guralp H, Iegorova V, Rodina M, Pšenička M. Elimination of primordial germ cells in sturgeon embryos by UV-irradiation. Biology of Reproduction 2018;221:3–564. doi:10.1093/biolre/ioy076.

[113] Smith LD. The role of a “germinal plasm” in the formation of primordial germ cells in *Rana pipiens*. Developmental Biology 1966;14:330–47.

[114] Tanabe K, Kotani M. Relationship between the amount of the “germinal plasm” and the number of primordial germ cells in *Xenopus laevis*. J Embryol Exp Morphol 1974;31:89–98.

[115] Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. PNAS 1994;91:11298–302.

[116] Majhi SK, Hattori RS, Rahman SM, Suzuki T, Strüssmann CA. Experimentally induced

depletion of germ cells in sub-adult Patagonian pejerrey (*Odontesthes hatcheri*). The 2009;71:1162–72. doi:10.1016/j.theriogenology.2008.12.008.

[117] Li M, Wang D. Gene editing nuclease and its application in tilapia. Science Bulletin 2017;62:165–73. doi:10.1016/j.scib.2017.01.003.

[118] Wong T-T, Collodi P. Inducible Sterilization of Zebrafish by Disruption of Primordial Germ Cell Migration. PLoS ONE 2013;8:e68455. doi:10.1371/journal.pone.0068455.

[119] Zhang Y, Chen J, Cui X, Luo D, Xia H, Dai J, et al. A controllable on-off strategy for the reproductive containment of fish. Sci Rep 2015;5:7614. doi:10.1038/srep07614.

[120] Nagasawa K, Ishida M, Octavera A, Kusano K, Kezuka F, Kitano T, et al. Novel method for mass producing genetically sterile fish from surrogate broodstock via spermatogonial transplantation. Biology of Reproduction 2018. doi:10.1093/biolre/ioy204.

Method	Donor species	Host species	Host sterility*	Phylogenetic distance**	Barrier	Migration/Settlement in host gonads	Sperm	Egg	Reference	
SPT	zebrafish	zebrafish	1	0	individual	yes	yes	yes	Saito et al., unpublished	
	zebrafish	zebrafish	4	0	individual	yes	yes	yes***	Saito et al., unpublished	
	Pearl danio	zebrafish	4	–	species	yes	yes	yes***		31
	goldfish	zebrafish	4	106.5	subfamily	yes	yes	no***		31
	loach	zebrafish	4	125	order	yes	yes	no***		31
	Rosy bitterling	zebrafish	1	66	family	yes	N/D	N/D	Goto et al., unpublished	
	medaka	zebrafish	1	229.9	class	yes***	N/A	N/A	Goto et al., unpublished	
	Japanese eel	zebrafish	1	274	class	yes	N/D	N/D		32
	sturgeon	goldfish	1	344	class	yes***	N/A	N/A		33
	goldfish	goldfish	4	0	individual	yes	yes	yes		34
	Rosy bitterling	goldfish	1	106.3	family	yes	N/D	N/D	Goto et al., unpublished	
BT	zebrafish	zebrafish	4	0	species	yes	yes	yes		30
	goldfish	zebrafish	4	106.5	subfamily	yes	N/D	N/D		30
	loach	zebrafish	4	125	order	yes	N/D	N/D		30
	loach	loach	1	0	individual	yes	N/D	N/D		20
	medaka	medaka	4	0	species	yes	yes	yes		24
BdT	zebrafish	zebrafish	4	0	species	yes	yes	yes		35
	crusian carp	goldfish	1	17	individual	yes	yes	yes		26
	goldfish	goldfish x common carp	3	–	–	yes	yes	N/D		29

PCT	zebrafish	zebrafish x Pearl danio	3	–	–	yes	yes	no		36
	common carp	goldfish	4	34	genus	yes	N/D	N/D	37, 38	
	medaka	medaka	3	0	individual	yes	yes	yes		39
	rainbow trout	rainbow trout	1	0	individual	yes	yes	yes		40
	rainbow trout	masu salmon	3	14.2	species	yes	yes	yes		41
	brown trout	rainbow trout	–	46	species	yes	N/D	N/D		42
	European grayling	rainbow trout	–	69	family	yes	N/D	N/D		42
	Chinese rosy bitterling	Chinese rosy bitterling	4	0	individual	yes	yes	yes		43
	blue drum	blue drum x white croaker	3	–	–	yes	yes	yes		44
	yellowtail	yellowtail	1	0	individual	yes	yes	yes		45
	Siberian sturgeon	Sterlet sturgeon	1	71	species	yes	N/D	N/D		46
	Chinese sturgeon	Darby's sturgeon	–	10.5	species	yes	N/D	N/D		47
	tiger puffer	grass puffer	3	4.22	species	yes	yes	yes		48
	Nibe croaker	chub mackarel	1	128	class	yes	N/A	N/A		49
GPT	zebrafish	zebrafish	2	0	individual	yes	yes	yes		50
	Jundia catfish	Nile tilapia	2	229.9	class	yes	yes	N/A		51
	Odontesthes bonariensis	Odontesthes hatcheri	2	–	species	yes	yes	yes		52
	tilapia	tilapia	2	0	individual	yes	yes	N/A		53

* Host sterility is classified as follow; 4, Nearly 100% sterility with germ cell depletion; 3, Nearly 100% sterility with meiotic or mitotic arrested germ cells; 2, partial depletion of germ cells; 1, without any treatment for sterilization.

** Estimated divergence times between donor and host were estimated by timetree.org (http://www.timetree.org/)

*** Donor PGCs disappeared after migration to gonadal ridges.

**** Germline chimeras were treated with ethynyl-estradiol (E2) for feminization.

Figure legends

Fig. 1. Methodologies and stages of germ cell transplantation in fish. A) Methodologies of PGCs, spermatogonia (SPG), and oogonia (OOG) transplantation reported in fish. B) Donor and host stages for germ cell transplantation for each method.

Fig. 2. The SPT method can provide unique experimental conditions for studying fish germ cells. The photograph demonstrates a chimera that has germ cells from pearl danio (green fluorescent protein-labeled cell) and zebrafish (red fluorescent protein-positive cell) at each side of the genital ridge region. To produce this chimera, a PGC from each donor was repeatedly transplanted into a single host.

Fig. 3. Schematic illustration of the migratory capacity of PGCs in goldfish and zebrafish embryos. The goldfish embryo is larger than the zebrafish embryo—assuming the migration path of PGCs is the same for each species, then goldfish PGCs migrate a greater distance than zebrafish PGCs. This probably explains why the percentage of goldfish and pearl danio PGCs that reached the gonadal region was higher than that of zebrafish after they were transplanted into zebrafish embryos.

Fig. 4. A flow chart of methods for the induction of germline chimera in fish. Methods of germ cell transplantation can be applied to the target species according to their material availability.

Fig. 5. A flow chart of the sterilization methods for surrogate hosts in fish. The sterilization methods listed in the figure can be applied to the target species according to their material availability. Note 1. Effective sterilization by ultraviolet irradiation was reported in sturgeon whose eggs undergo holoblastic cleavage. Note 2. Effective sterilization was reported in zebrafish using artificially fertilized eggs, and the rate of infertility was reduced when using naturally spawned eggs, Wong and Zohar [111].

Fig. 1

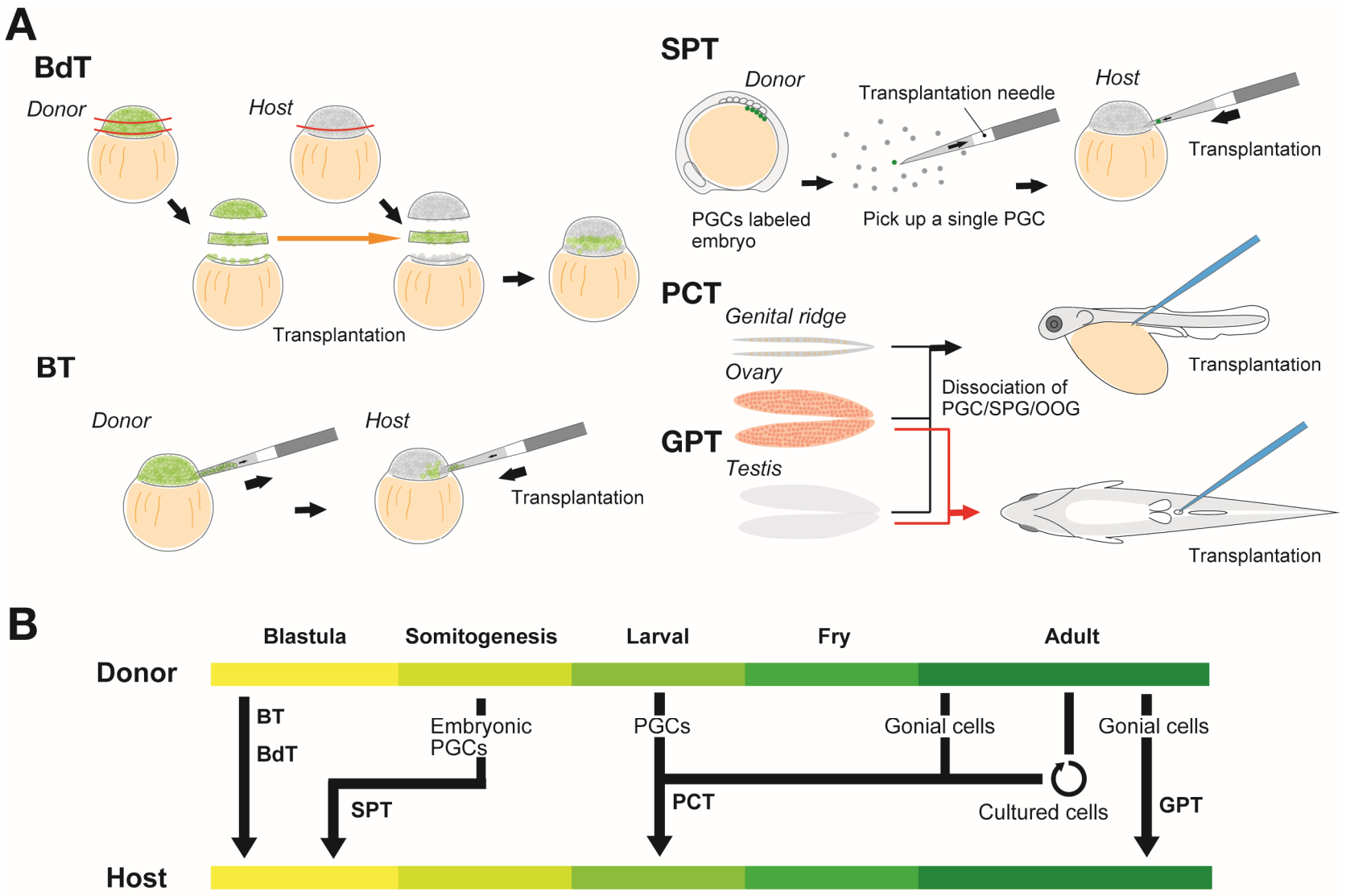

A
BdT
Donor
Host
Transplantation
BT
Donor
Host
Transplantation
SPT
Donor
Transplantation needle
Host
Transplantation
PGCs labeled embryo
Pick up a single PGC
PCT
Genital ridge
Ovary
Dissociation of PGC/SPG/OOG
Transplantation
GPT
Testis
Transplantation
B
Blastula
Somitogenesis
Larval
Fry
Adult
Donor
BT
BdT
Embryonic PGCs
SPT
PGCs
PCT
Gonial cells
Cultured cells
Gonial cells
GPT
Host

Fig. 2

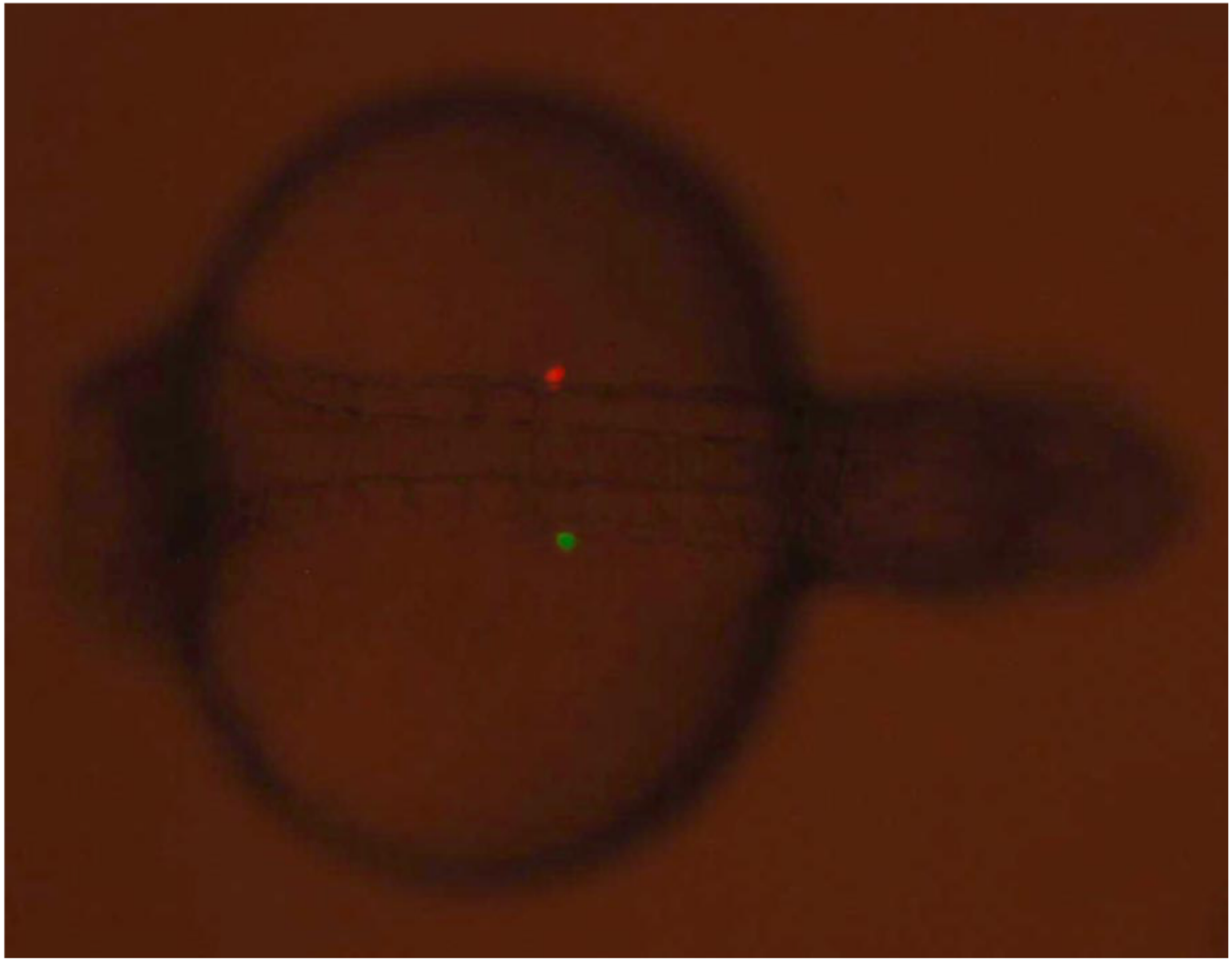
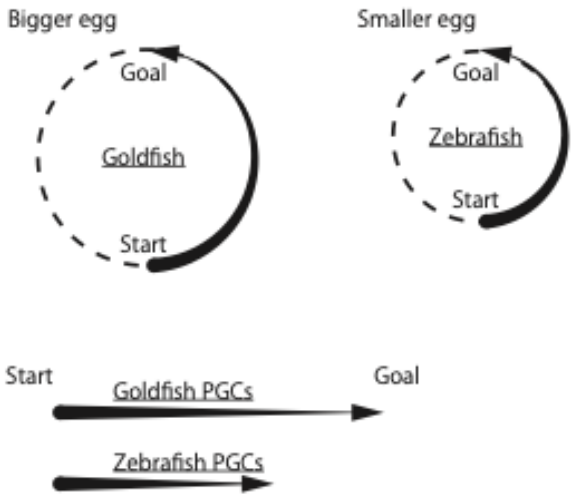


Fig. 3



Host sp.	Donor sp.	Approximate size of egg	Rate of germline chimera
zebrafish	zebrafish	0.6mm	30%
	Pearl danio	0.8mm	40%
	goldfish	1.0mm	60%

Fig. 4

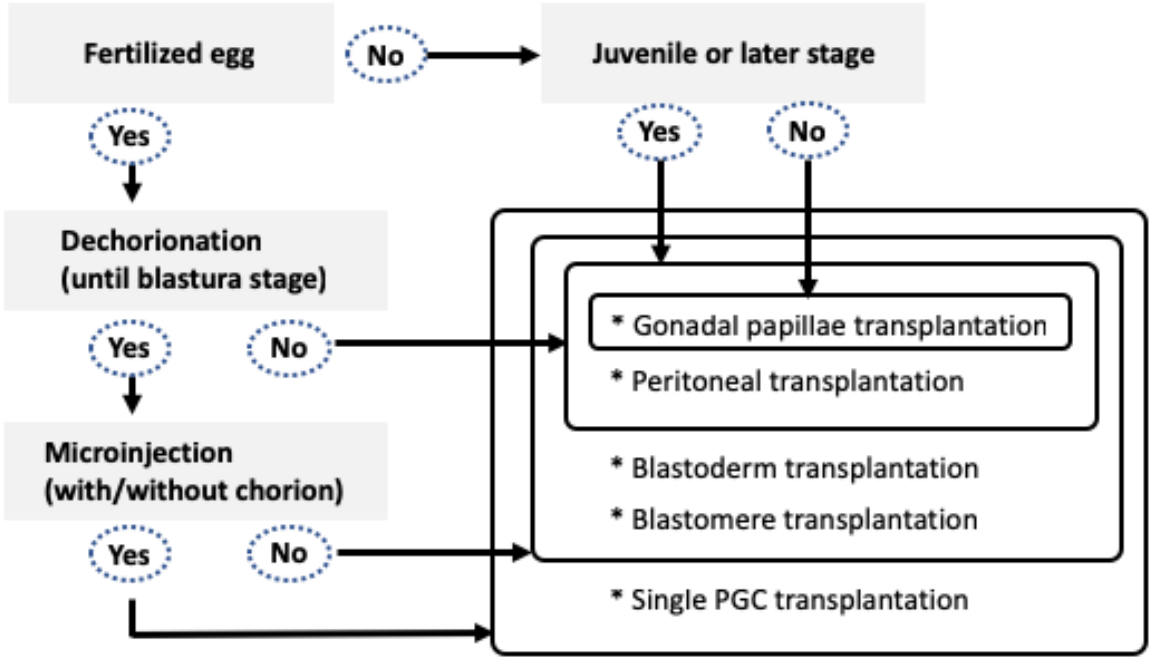

Fertilized egg
No
Juvenile or later stage
Yes
Yes
No
Dechorionation
(until blastura stage)
Yes
No
Microinjection
(with/without chorion)
Yes
No
* Gonadal papillae transplantation
* Peritoneal transplantation
* Blastoderm transplantation
* Blastomere transplantation
* Single PGC transplantation

Fig. 5

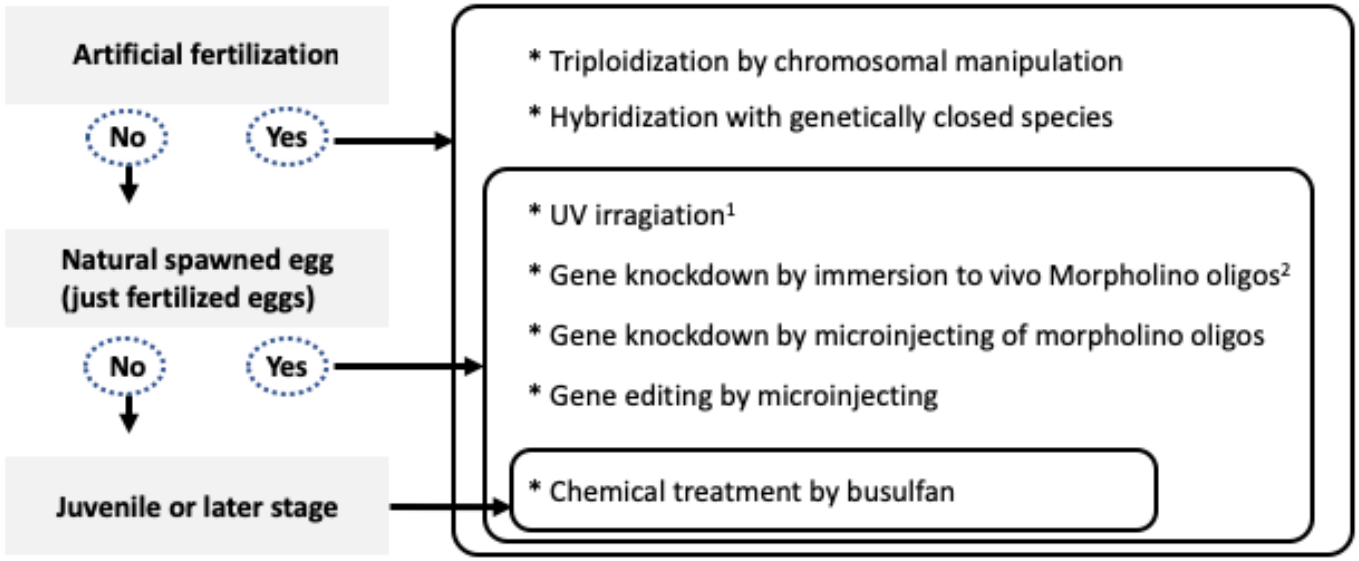

Artificial fertilization
No
Yes
Natural spawned egg
(just fertilized eggs)
No
Yes
Juvenile or later stage
* Triploidization by chromosomal manipulation
* Hybridization with genetically closed species
* UV irragiation[1]
* Gene knockdown by immersion to vivo Morpholino oligos[2]
* Gene knockdown by microinjecting of morpholino oligos
* Gene editing by microinjecting
* Chemical treatment by busulfan

Table 1

Method	Donor species	Host species	Host sterility*	Phylogenetic distance**	Barrier	Migration/Settlement in host gonads	Sperm	Egg	Reference
SPT	zebrafish	zebrafish	1	0	individual	yes	yes	yes	Saito et al., unpublished
	zebrafish	zebrafish	4	0	individual	yes	yes	yes****	Saito et al., unpublished
	Pearl danio	zebrafish	4	–	species	yes	yes	yes****	31
	goldfish	zebrafish	4	106.5	subfamily	yes	yes	no****	31
	loach	zebrafish	4	125	order	yes	yes	no****	31
	Rosy bitterling	zebrafish	1	66	family	yes	N/D	N/D	Goto et al., unpublished
	medaka	zebrafish	1	229.9	class	yes***	N/A	N/A	Goto et al., unpublished
	Japanese eel	zebrafish	1	274	class	yes	N/D	N/D	32
	sturgeon	goldfish	1	344	class	yes***	N/A	N/A	33
	goldfish	goldfish	4	0	individual	yes	yes	yes	34
	Rosy bitterling	goldfish	1	106.3	family	yes	N/D	N/D	Goto et al., unpublished
BT	zebrafish	zebrafish	4	0	species	yes	yes	yes	30
	goldfish	zebrafish	4	106.5	subfamily	yes	N/D	N/D	30
	loach	zebrafish	4	125	order	yes	N/D	N/D	30
	loach	loach	1	0	individual	yes	N/D	N/D	20
	medaka	medaka	4	0	species	yes	yes	yes	24
BdT	zebrafish	zebrafish	4	0	species	yes	yes	yes	35
	crusian carp	goldfish	1	17	individual	yes	yes	yes	26
	goldfish	goldfish x common carp	3	–	–	yes	yes	N/D	29
PCT	zebrafish	zebrafish x Pearl danio	3	–	–	yes	yes	no	36
	common carp	goldfish	4	34	genus	yes	N/D	N/D	37, 38
	medaka	medaka	3	0	individual	yes	yes	yes	39
	rainbow trout	rainbow trout	1	0	individual	yes	yes	yes	40
	rainbow trout	masu salmon	3	14.2	species	yes	yes	yes	41
	brown trout	rainbow trout	–	46	species	yes	N/D	N/D	42
	European grayling	rainbow trout	–	69	family	yes	N/D	N/D	42
	Chinese rosy bitterling	Chinese rosy bitterling	4	0	individual	yes	yes	yes	43
	blue drum	blue drum x white croaker	3	–	–	yes	yes	yes	44
	yellowtail	yellowtail	1	0	individual	yes	yes	yes	45
	Siberian sturgeon	Sterlet sturgeon	1	71	species	yes	N/D	N/D	46
	Chinese sturgeon	Darby's sturgeon	–	10.5	species	yes	N/D	N/D	47
	tiger puffer	grass puffer	3	4.22	species	yes	yes	yes	48
	Nibe croaker	chub mackarel	1	128	class	yes	N/A	N/A	49
GPT	zebrafish	zebrafish	2	0	individual	yes	yes	yes	50
	Jundia catfish	Nile tilapia	2	229.9	class	yes	yes	N/A	51
	Odontesthes bonariensis	Odontesthes hatcheri	2	–	species	yes	yes	yes	52
	tilapia	tilapia	2	0	individual	yes	yes	N/A	53

* Host sterility is classified as follow; 4, Nearly 100% sterility with germ cell depletion; 3, Nearly 100% sterility with meiotic or mitotic arrested germ cells; 2, partial depletion of germ cells; 1, without any treatment for sterilization.

** Estimated divergence times between donor and host were estimated by timetree.org (http://www.timetree.org/)

*** Donor PGCs disappeared after migration to gonadal ridges.

**** Germline chimeras were treated with ethynyl-estradiol (E2) for feminization.