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REVIEW ARTICLE

Application of surrogate broodstock technology in aquaculture

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Abstract

Surrogate broodstock technology facilitates the production of donor-derived gametes in surrogates, and comprises transplanting germ cells of a donor into recipients of a different strain or different species. The following applications of this technology are expected in the field of aquaculture: (1) the efficient and reliable production of offspring carrying superior genetic traits by transplanting donor germ cells from a single selected fish with superior traits into many recipient fish; (2) the reduction of the time required to breed fish by using a recipient species with a short generation time to produce gametes of a species with a long generation time; (3) the long-term storage of valuable species or strains as genetic resources by cryopreserving germ cells for transplantation; (4) the mass production of genetically sterile fish by transplanting germ cells of a donor fish that is sterile due to a mutation in the somatic cells into normal recipients without this mutation. It is expected that a combination of these techniques will greatly accelerate the breeding of aquaculture species. It is important to adapt surrogate broodstock technology to a wider range of fishery species and further improve the efficiency of donor-derived gamete production when using surrogate broodstock.

Keywords Germ cell transplantation · Germ-line stem cell · Spermatogonia · Cryopreservation · Sterilization

Methods to generate surrogate broodstock

Surrogate broodstock technology consists of producing donor-derived gametes in a surrogate fish (recipient individual) by transplanting germ cells of a donor into a recipient of a different strain or species. This technology can be facilitated by the transplantation of a cell suspension from the testis or ovary containing germline stem cells, which will eventually become sperm or eggs, respectively, into larvae immediately after hatching. Even though allogeneic or xenogeneic donor cells are transplanted into recipients, rejection can be avoided because the newly hatched larvae do not have a mature immune system and thus the ability to reject foreign substances (Takeuchi et al. 2004; Okutsu et al. 2007). In addition, donor-derived germline stem cells do not need to be transplanted into the testis or ovary of the recipient larvae. After transplantation into the intraperitoneal cavity by a fine glass pipette, they spontaneously migrate to the immature testis and ovary, into which they are incorporated and initiate spermatogenesis and oogenesis, respectively (Fig. 1) (Takeuchi et al. 2003; Okutsu et al. 2006). Furthermore, it is not necessary to purify the germline stem cells used for transplantation. When testis or ovary tissues are dissociated by proteinase in preparing the cell suspension for transplantation, only germline stem cells migrate to the recipient's genital ridges for incorporation, while the remaining cells eventually die in the abdominal cavity (Okutsu et al. 2006). Therefore, germline cell transplantation comprises an extremely simple microscopic operation using a stereomicroscope and a coarse motion micromanipulator.

We have successfully generated masu salmon *Oncorhynchus masou*-producing gametes of rainbow trout *Oncorhynchus mykiss* using this method (Takeuchi et al. 2004; Okutsu et al. 2007). The remarkable aspect of this technology is that female recipients produce functional eggs derived from donor cells after the transplantation of spermatogonial stem cells prepared from the donor testis (Okutsu et al. 2006) and male recipients produce functional sperm derived from the donor after the transplantation of oogonial stem cells prepared from the donor ovary (Yoshizaki et al. 2010a). Evidently, if the sex of the donor and recipient match, sperm

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Fig. 1 Microinjection of donor-derived germline stem cells into abdominal cavity of rainbow trout hatchlings. Intraperitoneally transplanted germline stem cells migrate to the genital ridges of the recipient, are incorporated, and initiate oogenesis or spermatogenesis

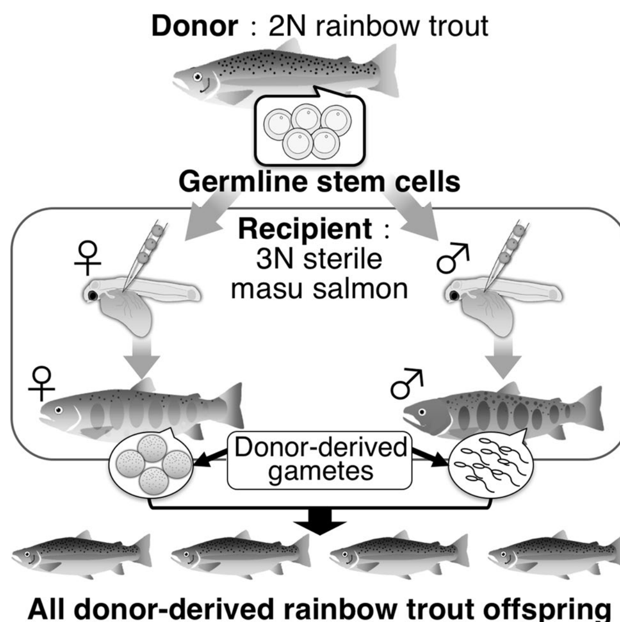

Fig. 2 Production of masu salmon surrogate broodstock and next generation of rainbow trout using germline stem cell transplantation. Spermatogonial stem cells from male rainbow trout were transplanted into the peritoneal cavity of newly hatched sterile triploid masu salmon; this resulted in the transplanted trout spermatogonia colonizing the gonads of the recipients, which eventually produced donor-derived eggs or sperm, depending on the sex of the recipients

will be generated by spermatogonial stem cells and eggs by oogonial stem cells. Accordingly, donor germline stem cells produce gametes based on the gender of the recipient, not their own gender (Yoshizaki et al. 2010b). When rainbow trout cells were transplanted into a wild-type masu salmon, the masu salmon recipients simultaneously produced gametes of both rainbow trout and masu salmon (Okutsu et al. 2008b). Moreover, it was possible to create a masu salmon that produced only rainbow trout gametes by transplanting germline stem cells derived from diploid rainbow trout into triploid sterile masu salmon (Fig. 2) (Okutsu et al. 2007). As an alternative to triploidization, sterile recipients lacking endogenous germ cells can be generated by gene knockdown or knockout of the *dead end* (*dnd*) gene, which is required for the maintenance of primordial germ cells (Saito et al. 2008; Linhartová et al. 2015; Wong and Zohar 2015; Yoshizaki et al. 2016; Wargelius et al. 2016; Li et al. 2017; Octavera and Yoshizaki 2018).

Similar to *dnd* knockdown or knockout, the generation of germ cell-less recipients has recently become possible by producing inter-species hybrids in the Sciaenidae. It has been confirmed that these recipients can maintain the ability to produce donor-derived gametes after germline stem cell transplantation (Yoshikawa et al. 2018a). In addition to the method of transplanting germline stem cells into newly hatched larvae, as described above, other methods have been developed. One consists of transplanting migrating primordial germ cells into the blastodisc at the blastula stage before their differentiation into germline stem cells (Saito et al. 2008, 2010). Another method consists of directly transplanting spermatogonial stem cells into an empty gonad after removing the endogenous germ cells of the parent fish by an alkylating agent such as busulfan (Majhi et al. 2009; Lacerda et al. 2010). Details of these methods can be found in other review articles (Lacerda et al. 2013; Yoshizaki and Lee 2018).

By using the above-mentioned intraperitoneal transplantation of germline stem cells, production of the next generation derived from transplanted donor germ cells using surrogate broodstock has been successfully employed in fish producing small eggs, such as yellowtail *Seriola quinqueradiata* (Morita et al. 2012, 2015), tiger puffer *Takifugu rubripes* (Hamasaki et al. 2017; Yoshikawa et al. 2018b), nibe croaker *Nibea mitsukurii* (Yoshikawa et al. 2017), and chub mackerel *Scomber japonicus* (Tani et al., unpublished data). Accordingly, in principle, it is possible to apply this method to various commercially important fish species, as long as they are oviparous. Thus, when considering the application of this technology, an important question is the extent to which the genetic distance between donor and recipient is acceptable. While further investigation is required to clarify the limits of genetic distance, on the basis of previous research, it is believed that recipients can stably produce both donor-derived eggs and sperm as long as they belong to the same genus (Bar et al. 2016), even if they are of different species (Table 1). Moreover, even if they do not belong to the same genus, at least the production of sperm is usually possible if the donor and recipient

Table 1 Intraperitoneal transplantation of xenogenic spermatogonial cells into hatchlings

Donor-recipient relationship	Donor	Recipient	Sterilization	Donor cell migration into genital ridges	Sperm production	Egg production	Reference
Intra-genus	Rainbow trout *Oncorhynchus mykiss*	Masu salmon *Oncorhynchus masou*	3N	O	O	O	Okutsu et al. (2007)
	Rainbow trout *Oncorhynchus mykiss*	Masu salmon *Oncorhynchus masou*	*dnd*-KD	O	O	O	Yoshizaki et al. (2016)
	Chinook salmon *Oncorhynchus tshawytscha*	Rainbow trout *Oncorhynchus mykiss*	3N	O	O	O	Namura et al., unpublished data
	Siberian sturgeon *Acipenser baerii*	Sterlet *Acipenser ruthenus*	–	O	–	–	Pšenička et al. (2015)
	Tiger puffer *Takifugu rubripes*	Grass puffer *Takifugu niphobles*	3N	O	O	O	Hamasaki et al. (2017)
Inter-genus	Rainbow trout *Oncorhynchus mykiss*	Japanese charr *Salvelinus leucomaenis*	3N	O	O	–	Okutsu et al. (2008a)
	Japanese charr *Salvelinus leucomaenis*	Rainbow trout *Oncorhynchus mykiss*	3N	O	O	O	Fujihara et al., unpublished data
	Yellowtail *Seriola quinqueradiata*	Jack mackerel *Trachurus japonicus*	–	O	O	–	Morita et al. 2015
	Brown trout *Salmo trutta*	Rainbow trout *Oncorhynchus mykiss*	–	O	–	–	Lujić et al. (2018)
	European grayling *Thymallus thymallus*						
	Slender bittering *Tanakia lanceolata*	Chinese rosy bitterling *Rhodeus ocellatus*	*dnd*-KD	O	O	O	Octavera et al., unpublished data
	Pacific bluefin tuna *Thunnus orientalis*	Chub mackerel *Scomber japonicus*	–	O	–	–	Yazawa et al. (2013)
	Mulloway *Argyrosomus japonicus*	Blue drum *Nibea mitsukurii* × White croaker *Pennahia argentata*	Interspecific hybridization	O	O	–	Takeuchi et al., unpublished data
Inter-family	Blue drum *Nibea mitsukurii*	Chub mackerel *Scomber japonicus*	–	O	–	–	Yazawa et al. (2010)
	Yellowtail *Seriola quinqueradiata*	Blue drum *Nibea mitsukurii*	–	O	–	–	Higuchi et al. (2011)
	Senegalese sole *Solea senegalensis*	Turbot *Psetta maxima*	–	O	–	–	Pacchiarini et al. (2014)
	Southern bluefin tuna *Thunnus maccoyii*	Yellowtail kingfish *Seriola lalandi*	–	O	–	–	Bar et al. (2016)
	Pacific bluefin tuna *Thunnus orientalis*	Blue drum *Nibea mitsukurii*	–	O	–	–	Ichida et al., submitted

3N Triploidization, *dnd*-KD *dead end* gene knockdown

belong to the same family (Morita et al. 2015) (Table 1). Recently, we also confirmed the successful production of donor-derived eggs by intergeneric transplantation in species of the Acheilognathinae (Octavera et al., unpublished data) and Salmonidae (Fujihara et al., unpublished data). Examples of the application of surrogate broodstock technology to various aquaculture techniques will be described in the following sections.

Acceleration of breeding by increased mating efficiency

In general, broodstock of marine fish are maintained in a large land-based tank, and fertilized eggs are collected using an egg collection net set at the water outlet of the tank. However, this method is not appropriate for efficient breeding methods that often require the mating of particular parent individuals carrying desirable genetic traits (i.e., selective breeding). In some fish species, males and females with superior traits can be artificially bred using a maturation induction technique using exogenous hormone administration. However, some marine fish are quite sensitive to handling stress, and there is also a risk of losing valuable parent fish during egg or sperm collection. Furthermore, the survival rate is usually low for fertilized eggs obtained by in vitro fertilization compared with naturally spawned eggs. Therefore, for fish species in which in vitro fertilization is difficult, one must rely on the spontaneous oviposition. In group-spawning marine fishes, it is not easy to obtain fertilized eggs from a small number of selected individual fish (in particular with a single male and single female in the tank). In extreme cases, mating between males and females with superior traits may become impossible because they do not reach maturity at the same time.

To overcome these drawbacks, germline stem cells from an individual fish carrying superior traits can be transplanted into a large number of recipient fish to generate a large number of females and males that produce gametes carrying genes associated with the superior traits. Group spawning of the resulting surrogate parent fish thereby leads to fertilization involving eggs and sperm derived from donors with superior traits (Fig. 3) (Morita et al. 2012). As a result, the efficiency of breeding will be much improved. The production of a next generation from surrogate parent fish that received donor-derived germ cells was successful in a group-spawning fish, the chub mackerel, by the administration of a gonadotropin-releasing hormone analogue (Tani et al., unpublished data).

As mentioned above, intra-species transplantation technology can achieve the production of genetically superior offspring from a donor. As in vitro fertilization has not yet been established for large-bodied fish such as the bluefin tuna *Thunnus orientalis*, intra-species transplantation is expected to be a potentially effective strategy for these. However, inter-species transplantation would have many more advantages. This latter technology is expected to be able to produce bluefin tuna from a small mackerel species such as the chub mackerel. Adult bluefin tuna are quite large and require 3–5 years to reach sexual maturity. In contrast, adult chub mackerel, which also belongs to the Scombridae, weigh 300 g and reach maturity in 1 year. Therefore, if the chub mackerel is able to produce eggs and sperm of bluefin tuna, the space, labor, and cost required for maintenance of the broodstock would be minimized. In addition, transplanting germline stem cells from bluefin tuna with superior traits into a small fish of the Scombridae would

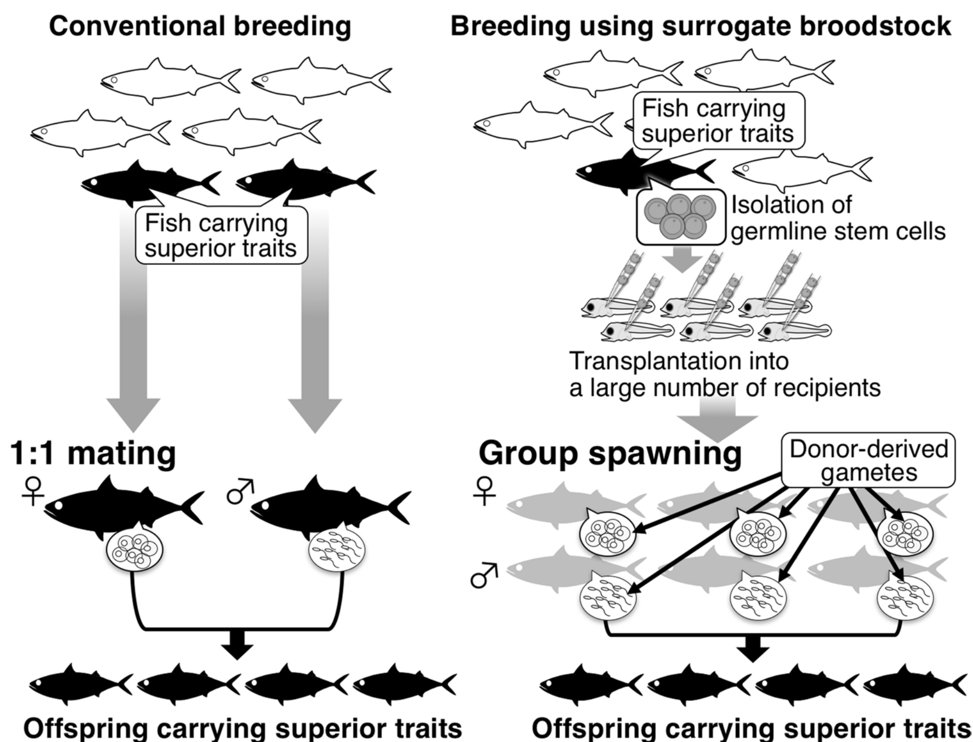

Fig. 3 Improvement in breeding efficiency of group spawner using surrogate broodstock. Fish carrying superior traits (black) and recipients (gray) are shown. Germ cell transplantation can produce a large number of recipients from a single donor fish with superior traits. This increases the probability of fish acquiring gametes carrying the donor-derived haplotype. Consequently, group spawning of the resulting recipients with mating between any individual female and male recipient leads to fertilization involving eggs and sperm derived from donors with superior traits

create genetically improved seedlings of bluefin tuna, which would be a far more efficient method than using bluefin tuna itself as a surrogate broodstock.

Acceleration of breeding by reducing the generation period

One of the major obstacles to breeding fish is the long generation time of many valuable aquacultural fish species. Mating experiments are indispensable in selective breeding programs, and there is strong demand to reduce the generation time for breeding species for the aquaculture industry and fish research. As shown by the example above, which describes the production of bluefin tuna from a small mackerel, it is possible to significantly shorten the period necessary for breeding using a fish species with a short generation time as surrogate broodstock. We have successfully produced eggs and sperm of tiger puffer more quickly by transplanting germline stem cells of this species into grass puffer *Takifugu niphobles* (Hamasaki et al. 2017). Generally, male tiger puffers require 2 years to mature, whereas female tiger puffers require 3 years to mature. Reportedly, both sexes of the grass puffer can mature within a year when the water temperature is controlled and photoperiod manipulated (Yoshiura et al., personal communication). Similarly, it is now possible to produce eggs and sperm of Chinook salmon *Oncorhynchus tshawytscha* (which normally require 3–5 years to mature) in 1 and 2 years, respectively, when using rainbow trout as surrogate broodstock (Namura et al., unpublished data).

The time required for breeding can be substantially reduced by the selection of individual fish with superior traits during the juvenile stages and by transplanting germline stem cells of the selected fish into a fish species with a short generation time. In recent years, selection using DNA markers has enabled faster and easier identification of target phenotypes based on the DNA analysis of larvae (Abdelrahman et al. 2017). Once the target juveniles or other young have been identified, their germline stem cells can be isolated and transplanted into a surrogate broodstock with a shorter generation time. Therefore, the establishment of a new, superior breed is then be possible in a much shorter time by repeating the above procedure for several generations. Thus, the combination of genome-based selection and surrogate broodstock technology may be a breakthrough for the future of fish breeding.

Cryopreservation of germline stem cells from a superior breed

Cryopreservation has not yet been fully adapted for fish because fish eggs are relatively large and rich in lipids and egg yolk (Mazur et al. 2008). Therefore, there is no technology available for permanently preserving the precious genetic resources of fish breeds, even though some breeds have been successfully developed over many years using a variety of breeding methods. In fact, although many clones and strains with unique genetic characteristics were created in various research stations of prefectural fisheries during the 1980s using various manipulation methods for chromosome sets, many fish breeds have already been lost due to the difficulty of maintaining these clones and strains.

Cryopreservation of germline stem cells can be easily performed in liquid nitrogen because the cells are small (approximately 10 µm) and do not contain much lipid or egg yolk. A method of freezing the testis of immature individuals (containing a large number of immature spermatogonia) in liquid nitrogen is well established, making it theoretically possible to permanently store germline stem cells within the testis in a frozen state (Yoshizaki et al. 2011; Yoshizaki and Lee 2018). In fact, our group showed that there was no decline in survival rate even after we thawed the testis of rainbow trout 5 years after freezing them in liquid nitrogen (Lee et al. 2013, 2016a). Also, it has already been shown that gametes develop normally in the gonads of surrogate fish and that sperm and eggs can be produced from frozen cells even when the cryopreserved cells are thawed and transplanted into recipient fish (Fig. 4) (Lee et al. 2013, 2015). Similarly, it is now possible to cryopreserve ovaries and to isolate and transplant germline stem cells derived from them

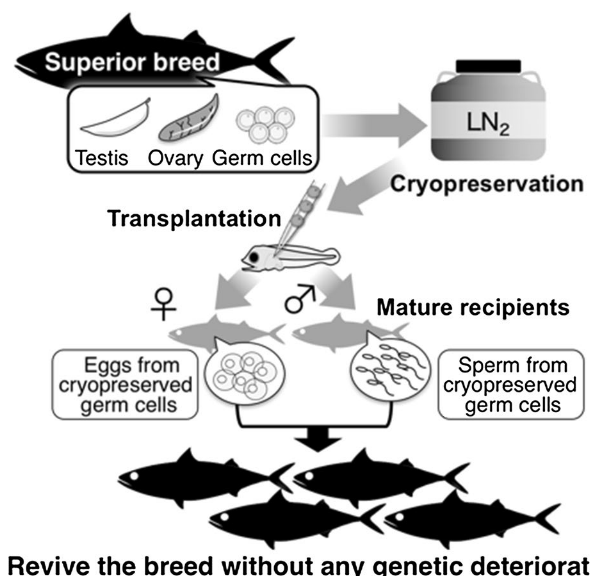

Fig. 4 Long-term preservation of superior fish breeds by freezing germline stem cells. Fish carrying superior traits (black) and recipients (gray) are shown. Germline stem cells can be maintained semi-permanently in liquid nitrogen. Frozen germline stem cells are transplanted into recipients to obtain functional gametes. By mating male and female recipients, fish breeds carrying superior traits can be regenerated by using frozen genetic material

(Lee et al. 2016b). Cryopreservation is a powerful method for preserving precious genetic resources as this technology requires no special and expensive equipment and is feasible as long as liquid nitrogen and cryo-containers are available. Because a cryopreservation technique for fish eggs is not yet available, a combination of cryopreservation and transplantation of germline stem cells could be extremely important for preserving the genetic resources of endangered species [details of these techniques can be found in other review articles (Yoshizaki et al. 2011; Yoshizaki and Lee 2018; de Siqueira-Silva et al. 2018)].

Producing sterile fish

After creating a superior fish breed via selective breeding, there is a risk that the next generation (seedlings with excellent traits) could be mass-produced, even unintentionally, by a third party by breeding first-generation seedlings and raising them to sexual maturity. The resulting seedlings could be sold as "pirated" versions of the original seedlings created with a great deal of effort over a long period of time. This would make it difficult for breeders to profit from their work. To solve this problem, it will be necessary to develop measures for sterilizing seed before selling it. Triploids created from chromosome manipulation would be major candidates for this (Piferrer et al. 2009); however, triploidization leads to a reduction in survival rates, and the triploid rate itself is not very high in some fish species (especially in marine species).

Gene knockdown techniques that specifically suppress the translation of a target gene and gene knockout techniques that disrupt target gene sequences have been recently developed in fishes (Huang et al. 2012; Zhu and Ge 2018). It is possible to create sterile fish by inhibiting the functioning of *dnd* genes, which are necessary to maintain primordial germ cells (described above as a way to generate surrogate broodstock). However, the gene knockdown and knockout techniques require individual microinjection of the antisense oligonucleotide and guide RNA, together with Cas9 protein, into fertilized eggs under a microscope. This, however, is not a practical method because gametes cannot be obtained from sterile individuals produced by these methods and microinjection into the fertilized egg is required every time to produce sterile fish.

Nagasawa et al. (2018) developed a method to mass produce sterile individuals using surrogate broodstock. Oogenesis of fish generally progresses when ovaries receive stimulation from follicle-stimulating hormone (FSH) secreted from the pituitary gland (Clelland and Peng 2009). The receptor for FSH is expressed not in germ cells but in supporting cells of the ovaries surrounding the germ cells. Female Japanese medaka with a homozygous mutation in the FSH receptor gene are sterile (Murozumi et al. 2014). However, male mutant medaka mature normally (Murozumi et al. 2014). Thus, we isolated germ cells from mutant (sterile) medaka and transplanted them into surrogate parent medaka that carried a wild-type FSH receptor. Because the receptor gene is dispensable for the germ cell itself, transplanted germ cells in the ovary of surrogate parent medaka are nursed by the ovarian supporting cells of the recipient and eventually produce normal eggs. When we fertilized these eggs with sperm of a pseudo-male of a FSH-receptor-mutant medaka (masculinized XX genotype medaka), all of the next generation was XX (and all-sterile females). Thus, it was possible to mass produce sterile individuals with disrupted FSH receptor genes (Nagasawa et al. 2018). In principle, this method can be applied to a wide variety of aquaculture fish species. If we could identify a gene that makes both males and females sterile via gene knockout, it would be possible to mass produce sterile fish by only mating males and females of the surrogate broodstock that carry the knocked-out gene (Fig. 5).

The above-described approach for disrupting the somatic genes related to gonadal maturation (via gene editing) is useful for preventing not only the property loss of superior breeds but also escaped fish from fish farms from breeding in the wild. Salmon escape is of concern because the escaped fish return to rivers to breed with wild populations, which disrupts the genetic composition of wild stocks (Glover et al. 2013). Indeed, the escape of Atlantic salmon has become an environmental problem in Norway and elsewhere. The mass production of genetically sterile individuals can reduce the negative impacts of aquaculture on natural ecosystems. The commercialization of genetically modified salmon began in Canada in 2017 (Waltz 2017), and there has been research on applying genome-editing technology to the breeding of farm-raised animals (including fish). We think that the sterilization technique described here could be extremely effective in preventing genetically modified and genome-edited individuals from crossbreeding with wild fish when they escape from fish farms.

Conclusion

In summary, surrogate broodstock technology can be useful to accelerate breeding and preserve the genetic information of broodstock. It can also be used to prevent the unauthorized production of pirated fish seedlings. Surrogate broodstock technology does not artificially manipulate cellular contents (unlike gene recombination or nuclear transfer technologies), which could make it advantageous for application on an industrial scale. However, several problems still have to be resolved before surrogate broodstock technology can be applied to the aquaculture of fish. Some fish

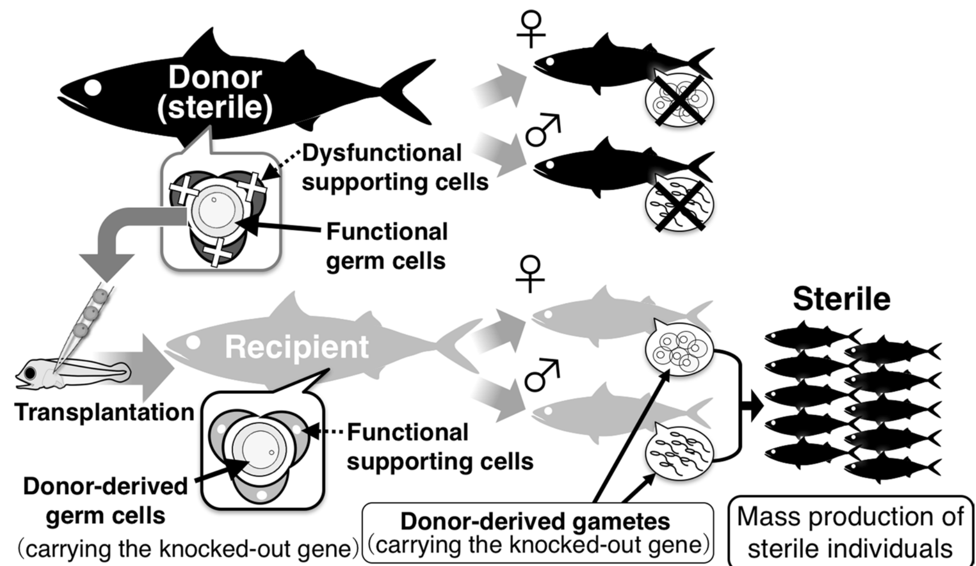

Fig. 5 Mass production of genetically sterile fish by surrogate broodstock technology. Donor (black) and recipients (gray) are shown. Although mutations in supporting cells cause sterility in donor fish, these fish carry functional germ cells. If germ cells from the mutant (sterile) donor are transplanted into wild type recipients carrying functional supporting cells, the transplanted germ cells are nursed by the supporting cells in the gonad of the recipient and eventually produce gametes carrying the mutation

species possess low concentrations of germline stem cells in their genital glands. In such species, the use of maturing young fish or immature adult fish (rather than fully mature adult fish) will allow the easy and efficient transplantation of germ cells because the concentration of germline stem cells is relatively high in the gonads of these fish. However, if younger fish are not available and one must use mature adults carrying large numbers of developed germ cells, germline stem cells must be enriched prior to transplantation. Several methods for isolating cell populations containing high concentrations of transplantable germline stem cells from mature testis have recently been developed by using a cell sorter (Kise et al. 2012; Hayashi et al. 2014; Ichida et al. 2017). Further, monoclonal antibodies that specifically recognize spermatogonia containing a high concentration of germline stem cells have been recently generated (Hayashi et al., unpublished data). The use of such antibodies concentrates transplantable cells via magnetic activated cell sorting without the need for any expensive equipment, including a cell sorter.

Another potential problem with transplanting germ cells is that the efficiency of transplantation can vary with the season, e.g., recent research on adult rainbow trout showed that clear seasonality exists in the amount of transplantable spermatogonia produced by their gonads (Sato et al. 2017). Hence, to maximize germ cell transplantation efficiency, it is important to take into account the time of year when germline stem cells accumulate in the gonads of adult donor fish.

A number of the technologies discussed above have recently become useful for both freshwater and marine fishes. We are hopeful that these technologies will be applied to the breeding of a wider range of aquaculture species in the near future.

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