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Progress in Infertility Control Technology of Fish

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Abstract

Infertility control of fish has been a significant research problem concerning many aquatic breeders. It is necessary to develop infertility control technology for fish to solve the ecological safety problems of existing transgenic fish with qualified characteristics. We reviewed here the implementation of intensely studied available fish infertility control technologies (e.g., triploid technology and antisense RNA technology), kid/kis system, *Ntr*/Met system, and Gal4/UAS system. Moreover, prospects in infertility control and technological development of fish are disclosed by combining relevant and associated studies.

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Introduction

Fish are an essential type of high-quality protein source that people depend on for survival and are the final wild food source (Holmlund and Hammer., 1999). Sustainable development of the fishery industry has become a globally themed, important focus for research (Pauly et al., 2002). With an increasing global population, fishing activities are increasingly challenged to meet human demands for aquatic products and threaten the sustainable supply of natural resources (Neubauer et al., 2013). In this case, aquaculture is viewed as the only way to meet the increasing demands for aquatic livestock proteins (Cressey, 2009). New cultured fish with good characteristics are key to future sustainable aquaculture development. Fish infertility-controlled breeding not only uses energy for reproductive development during physical growth and accelerates the growth rate of fish but can solve many problems caused by the early maturity of fishes in the aquaculture process.

Artificial breeding, introduction, and crossbreeding are traditional methods for evolving new species with good characteristics (Shelton and Rothbard, 2006; Hulata, 1995). However, traditional breeding methods significantly restrict the development of the fish breeding industry due to the ultra-long breeding years. Transgenic technologies can break interspecies limitations and quickly get new species with good characteristics. So far, nearly 40 transgenic fishes with good traits, such as fast growth, high bait utilization, and strong resistance to disease and stress, have resulted from transgenic operations and almost cover all essential cultured fish in the world (Yan et al., 2021; Wang et al., 2010). Worldwide, only transgenic salmon has been approved by the Food and Drug Administration (FDA) to launch into the market after being investigated for more than 20 years. In the aquaculture process, physical, geographic, and biological isolations are designed to prevent contact of transgenic salmon with the natural environment. Therefore, ecological safety is the primary barrier to marketing transgenic fish. In other words, people worry that transgenic fishes may escape into the natural environment and cause ecological disturbances, resulting in the extinction of the same species or other fish (Devlin et al., 2006). Therefore, mitigating such complications has become the current research focus. Solving these problems is an essential prerequisite for commercial aquaculture of transgenic fish. The development of infertility-control technology for fish may be an effective tool for evolving commercial aquaculture (Bazaz et al., 2020).

1. Triploid strategy controls infertility of fish

The artificial triploid-inducing method primarily includes physical and chemical methods. The former includes mainly temperature and hydrostatic shocks, while the latter refers to triploid production through processing by chemicals, such as colchicine or cytochalasin B (Bazaz et al., 2020). The triploid efficiency in some fish can reach 100% in the laboratory, such as *Scophthalmus maximus* (Piferre et al., 2003) and *dicentrarchus labrax* (Peruzzi and Chatain, 2000). However, some embryos remain diploid in most fish in the laboratory after artificial induction (Bazaz et al., 2020), with coexistence between diploid cells and triploid cells found in some embryos after induction. If these diploid cells participate in developing gonads, the resulting embryos may develop into fertile adults (Benfey, 1999). Notably, it is challenging for artificially induced triploid efficiency in large commercial production to reach 100% (McGeachy et al., 1995). Artificially induced triploid efficiency changes with variations between fish species, egg quality, and induction methods (Wong and Van Eenennaam, 2008).

Although chromosome pairing and separation disorder may occur when artificially induced triploid fish form gametes through meiosis, enough steroid hormones exist in triploid male fish to promote the formation of partial or complete testes and generate sperm. This phenomenon occurs in triploid of several fish, such as Gadus *morhua* (Feindel et al., 2010), *Oncorhynchus mykiss* (Kobayashi et al., 2008), *Tilapia mossambica* (Brämick et al., 1995). For triploid female fish, the ovaries of triploid female fish are in a childish development state due to low blood levels of steroid hormones (such as testosterone and estradiol) (Kobayashi et al., 2008).

However, many studies report that some triploid female fish may generate a few mature eggs (Matsubara et al., 1995; Schafhauser-Smith and Benfey, 2001).

Hybridization of tetraploid and diploid cells is another method of achieving triploid fish. These triploids have better infertility characteristics than triploids induced directly. The tetraploid with stable inheritance is necessary for this method, and tetraploid induction is like the induction of triploids. A fertilized embryo in the first mitosis stage via physical or chemical methods stops cell division under chromosome doubling, thus generating monoplasts containing four groups of chromosomes. These embryos have four groups of chromosome cells, causing tetraploid organisms. Since tetraploids contain an even number of chromosomes, normal diploid gametes will form (Larsen and Brett, 2005). However, the efficiency of tetraploid induction is far lower than for triploids, with the survival rate of tetraploids decreasing significantly, accompanied by slow growth and reduced reproduction ability (Donaldson and Devlin, 1996; Pandian and Koteeswaran, 1998).

Therefore, current investigations only realized the induction of tetraploid organisms in some fish. Allotetraploid with bisexual fertility can be produced through distant hybridization, which then is hybridized with diploid cells to produce an infertile triploid fish. For example, Liu et al. (2010) created allotetraploid carp groups through the distant hybridization of male red crucian carp and female cyprinoids. Combining androgenesis and gynogenesis resulted in several species of improved allotetraploid crucian-carps. Many 100% infertile triploid offspring "cyprinoid" have resulted through hybridization between the improved female allotetraploid crucian carp and male *Cyrinus carpio* (Liu, 2010). However, with limited application ranges, not all economic fish can yield tetraploid organisms through distant hybridization.

Due to the imbalanced chromosome composition of triploid fish, it is challenging to develop mature gonads, while energy for reproductive development can be directed to physical growth. The growth rate of triploid fish should be higher than diploid fish. However, this does not always happen. In some fish, the growth rate of triploid fish is more elevated than diploid fish, such as *Scophthalmus maximus* (Aydin, 2021) and European catfish (Krasznai and Marian, 1986). In some other fish, the growth rate of triploid fish is lower than in diploid fish, such as *Dicentrarchus labrax* (Felip et al., 2001), *Salmo gairdneri* Richardson (Solar et al., 1984), and *Salmo salar* (Galbreath and Thorgaard, 1995). In some fish, triploid fish also show some characteristics disadvantageous for commercial production, such as decreased resistance to disease (Ozerov et al., 2010), reduced resistance to stress (Fraser et al., 2012), and deformations (Oppedal et al., 2003).

Artificially induced triploids are the current principal method for controlling fish reproduction. The above analysis highlights the many uncertainties arising from triploid induction, such as deformations, survival of triploid fish, and the development of some characteristics. Hence, this strategy is inapplicable to the fertility control of all cultivated fish, particularly commercially.

2. Antisense RNA technology control of fish infertility

Regulating the expression of reproductive development-related genes through transgenic technology is a standard method of controlling fish fertility. The practical application primarily involves two aspects. Firstly, antisense RNA technology inhibits endogenous gene expression, thus realizing fish infertility. Secondly, infertile individuals recover fertility by feeding or injecting exogenous hormones, thus enabling them to keep advantageous and preferred characteristics.

When antisense RNA technology is applied to control fish reproduction, *GnRH* is often used as a target gene, mainly because fish reproduction is regulated by neuroendocrinology. The hypothalamic-pituitary-gonadal axis (HPG axis) is the primary neuroendocrine pathway in fish. The hypothalamus secretes GnRH, also stimulating hypophysis to secrete gonadotropin, which circulates in the blood to reach the gonads. Meanwhile, gonads are facilitated to secrete steroid hormones, thus regulating gonadal development (Cao et al., 2014). *GnRH* functions at the top

of the HPG axis. If *GnRH* is inhibited, the entire HPG axis is affected, thus significantly influencing reproduction. Mutation of *GnRH* may lead to complete infertility of mice, and treatment with native *GnRH* or supplementing exogenous androgens can enable infertile mice to recover fertility (Mason et al., 1986). Such a central role in reproduction is why *GnRH* is focused upon in investigations of fish fertility control. Uzbekova et al. successfully inhibited the expression of GnRH3 in rainbow trout using antisense RNA technology for the first time (Uzbekova, 2000). Maclean and co-workers drove transcription of *GnRH3* antisense fragments in *Tilapia mossambica* by using the β-actin promoter of cyprinoid and inhibited expression of endogenous *GnRH3* in *Tilapia mossambica* in the Nile. Some completely infertile individuals resulted from the P0 generation (Maclean et al., 2002). Hu et al. (2006) drove transcription of *GnRH3* antisense fragment in cyprinoid by the cyprinoid β-actin promoter and inhibited expression of endogenous *GnRH3*. The gonadal tissues were not developed in about 30% of individuals in the P0 generation.

In conclusion, fertility control of fish can be realized by inhibiting the expression of endogenous *GnRH* using antisense RNA technology. However, recovering the fertility of infertile organisms using in vitro supplementation of hormones has a poor outcome due to gonadal tissue shrinkage or completely disappearing once fish become infertile, making it extremely difficult to rebuild perfect gonadal tissues. Currently, no better method exists to realize fertility recovery of infertile transgenes, which may be why the fish fertility control method based on antisense RNA technology is not promoted.

3. Infertility control of fishes based on cytotoxic protein

To some extent, control over fish reproduction controls the survival of germ cells in fish. Fish fertility can be successfully maintained if fish germ cells are specifically removed. At present, specific expression of toxalbumin or "suicide genes" in a type of cell is an effective method to eliminate these cells. For example, Saito et al. drove the expression of Diphtheria toxin (DT) molecules in hepatic cells by using hepatocyte-specific promoters (albumin-promoter), eliminating the hepatic cells of mice effectively (Saito et al., 2001), yielding an excellent mice model to study liver diseases. The collaborative manipulation of cytotoxic protein Kid and detoxification protein Kis can effectively eliminate a type of cell. The strategy is to express cytotoxic protein Kid in cells which has to be eliminated but express Kis in other cells to protect cells that are not necessary to be eliminated (de la Cueva-Mendez et al., 2003; Slanchev et al., 2005). Smith et al. (2007) drove the expression of virus toxalbumin factor M2 by using a specific promoter, eliminating a type of cell and macrophages in the hearts of *Xenopus laevis*. Although cells can be eliminated effectively and precisely by toxalbumin, it is challenging to eliminate cells without influencing nearby cells and establish a stable family since they have extreme toxicity (Li et al., 2002). Moreover, such toxalbumin is more inapplicable to fertility control of economic fish, which provide food proteins.

4. Infertility control of fishes based on *Ntr*/Met system

Germ cells in fishes can be eliminated using *Ntr* (nitroreductase) encoded in *Escherichia coli* and the substrate Met (metronidazole). The principle of this method is to build a transgenic family in which a gonadal-specific promoter drives the expression of Ntr. Secondly, individuals of this family were processed using a specific concentration of Met solution. Met absorbed by the fish is decomposed into a cytotoxin by NTR with particular expression in gonads. This cytotoxin can kill germ cells, thus inducing infertility in fish. Hsu et al. drove the expression of *Ntr* using a testis-specific expression promoter and established a family of transgenic zebrafish. Two weeks after fertilization, the transgenic embryo was processed by Met, producing gonad abortive transgenic male zebra fishes (Hsu et al., 2010). Hu et al. drove the expression of *Ntr* using an ovary-specific expression promoter and established a family. Met treatment of transgenic embryos resulted in gonad abortive transgenic female zebrafish. This method has been used to afford transgenic ornamental zebrafish fertility control (Hu et al.,

2010). Unfortunately, using Met solutions may generate cytotoxin in fish bodies, which is inappropriate for large-scale promotion from the environmental protection and food safety perspectives.

5. Infertility control of fishes based on tet-on/off system

Wong and Van Eenennaam (2008) proposed a method to control the fertility of fishes based on a tet-on/off system. The basic principle of this method involves several steps. Firstly, a family of transgenic fish is established, containing two promoters. One promoter drives the expression of the tetracycline response transcription activator (tTA). The other promoter comprises a tTA response element (TRE) and a mini CMV promoter. It can only drive downstream gene transcription after activating mini CMV promoters under the combination of tTA and TRE. However, it cannot activate transcription of downstream genes without tTA or under the failure of tTA. Tetracycline can specifically bind with tTA, making tTA unable to bind with TRE. Therefore, tTA can drive the transcription of downstream genes of one promoter when there is no tetracycline in the aquaculture environment. If downstream links of this promoter target an antisense fragment or ribozyme of essential genes for reproductive development, it can realize the infertility of fish. When there is tetracycline in an aquaculture environment, tetracycline may combine with tTA to make it unable to bind with TRE. As a result, the promoter, containing a tTA response element (TRE) and a mini CMV promoter, becomes silent. Hence, this transgenic fish is fertile in an environment with tetracycline but infertile in an environment without tetracycline, enabling the fertility control of fish (Wong and Van Eenennaam, 2008).

Kishimoto and co-workers (1997) inhibited the expression of *zBMP2* by using one promoter-driven tTA expression, which has activity in the embryo development stage of zebrafish, including dsRNA transcription of bone morphogenetic protein gene (*zBMP2*) driven by TRE and a mini CMV promoter. *zBMP2* plays an essential role in the embryo development stage of zebrafish, and its expression anomalies may lead to embryo death (Kishimoto et al., 1997). However, all embryos were not killed in an environment lacking tetracycline. Adding inducers into the aquaculture water incurs high cost and low efficiency and may influence the environment for large aquaculture fish.

6. Infertility control of fishes based on Gal4/UAS system

Since there is no homologous gene of the GAL family in fish, the GAL4/UAS system will not be influenced by endogenous genes in fish, enabling accurate induction realization (Osterwalder et al., 2001; Distel et al., 2009). Families containing a transgenic fish family of GAL4 and UAS-driven target gene expression need to be established. GAL4 can accurately induce the transcriptional expression of the UAS downstream gene in the hybrid offspring of these two families. This gene may realize fish infertility if it is essential for reproductive development. Such a reproduction control strategy for fish can control the reproduction of transgenic fish, thus solving their potential ecological safety problems and protecting the intellectual properties of transgenic fish. Using the GAL4/UAS induction system, Zhang et al. established the TG1 family of CMV promoter-driven GAL4 expression and the TG2 family of 5xUAS driven transcription of antisense RNA fragment of the *dnd* gene in zebrafish, respectively. Both TG1 and TG2 can reproduce offspring. However, GAL4 induces transcription of antisense RNA fragment of 5xUAS driven *dnd* gene in the hybridization offspring of TG1 and TG2, inhibiting expression of endogenous *dnd*.

Consequently, the primary germ cells (PGCs) develop migration disorder and apoptosis in the early development of hybridization embryos. The quantity of PGCs migrating to the germinal ridge decreases significantly, or even no PGCs migrate to the germinal ridge. The larva with a significant reduction and absence of PGCs finally grow into infertile adults. As a result, the goal of infertility induction of hybridization offspring from fertile parents is realized.

However, this study, unfortunately, fails to realize the complete infertility of all hybridization offspring (Zhang et al., 2015).

Conclusion

Fertility control of fish has been a research hotspot for aquaculture breeders. Many fertility control technologies for fish have been developed, such as triploid, antisense RNA, and various systematic strategies and technologies. These technologies have unique advantages; they can induce infertility or decrease fertility in some specific fish. Nevertheless, they all have disadvantages for application in all fish. To address these problems, fertility control in zebrafish is studied systematically by using the mature CRISPR/Cas9 technology. The implementation program is shown in Fig.1. The drive expression of *Cas9* by the specific kop promoter of germ cells is activated, establishing a family. The drive transcription of gRNA by U6 promoters is activated, establishing a family. In hybridization, the offspring of two families, genes, or some target sites related to reproductive development are knocked-out specifically to realize infertility.

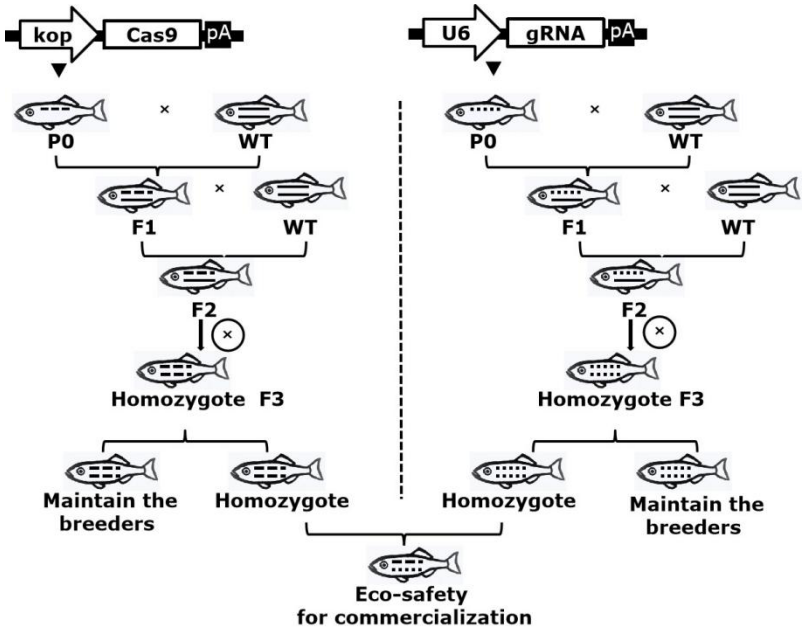

Figure 1 Infertility control strategy of fishes

When germ cell migration and essential genes for survival (*dnd*) are chosen as the target genes, it can only decrease the reproductive capacity of hybridization offspring since the knockout efficiency is less than 100% and 2/3 of mutation types are non-frameshift mutations. However, when repetitive sequences are used as the target sites, all hybridization offspring realize complete infertility (data unpublished). Of course, further studies are required to investigate whether CRISPR/Cas9 technology can be used in all economic fishes and the effect.

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