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Suppression and restoration of primordial germ cell marker gene expression in channel catfish, *Ictalurus punctatus*, using knockdown constructs regulated by copper transport protein gene promoters: Potential for reversible transgenic sterilization

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ABSTRACT

Complementary DNA overexpression and short hairpin RNA interference approaches were evaluated for decreasing expression of primordial germ cell (PGC) marker genes and thereby sterilizing channel catfish, *Ictalurus punctatus*, by delivering knockdown constructs driven by a constitutive promoter from yeast and a copper transport protein gene into fish embryos by electroporation. Two PGC marker genes, *nanos* and *dead end*, were the target knockdown genes, and their expressions, along with that of an off-target gene, *vasa*, were evaluated temporally using real-time polymerase chain reaction. Copper sulfate was evaluated as a repressor compound. Some of the constructs knocked down PGC marker gene expression, and some of the constructs were partially repressed by application of 0.1-ppm copper sulfate. When the rate of sexual maturity was compared for three-year-old broodfish that had been exposed to the sterilizing constructs during embryologic development and controls that had not been exposed, several treatments had reduced sexual maturity for the exposed fish. Of two promoter systems evaluated, the one which had been designed to be less sensitive to copper generally was more effective at achieving sterilization and more responsive to repression. Knockdown constructs based on 3′ *nanos* short hairpin RNA interference appeared to result in the best repression and restoration of normal sexual maturity. We conclude that these copper-based systems exhibited good potential for repressible transgenic sterilization. Optimization of this system could allow environmentally safe application of transgenic technology and might be applicable to other applications for aquatic organisms.

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1. Introduction

Transgenic technology has been used to improve aquaculture stocks for traits such as growth, color, body composition, disease resistance, tolerance to heavy metal exposure, and survival at low temperature, as well as for production of pharmaceutical proteins [1,2]. The great potential of transgenic technology was reported in transgenic coho salmon (*Oncorhynchus kisutch*) with an average 11-fold increase of growth compared to nontransgenic controls [3], and with 30-fold improvement of growth of mud loach (*Misgurnus mizolepis*) [4], growth that probably could never be achieved by traditional selective breeding. Channel catfish (*Ictalurus punctatus*) growth also was increased with growth hormone gene transfer [5], and bacterial disease resistance improved with cecropin gene transfer [6]. Transgenic alteration of the nutritional characteristics of fish could benefit consumers, and it is now possible to directly alter body composition *via* transgenesis; zebrafish (*Danio rerio*) and common carp (*Cyprinus carpio*) transfected with *β-actin*-salmon desaturase genes had enhanced levels of omega-3 fatty acids, docosahexaenoic acid, and eicosapentaenoic acid in their flesh [7,8].

However, a major element of concern over the use of transgenic technology in aquaculture revolves around the scenario of escape of transgenic fish from confinement. Escape could allow the establishment of the transgenic genotype in native fish populations, with an associated risk of environmental, ecological, and genetic damage. As a result, considerable work has been done to develop confinement technologies, including physical confinement, physicochemical confinement, genetic confinement, and operation management practices [9,10]; however, none of the technologies currently available are ideal [11].

Transgenic sterilization offers a partial solution by providing reproductive confinement. Zebrafish were transgenically sterilized by using a heat shock protein 70 promoter to express stromal-derived factor 1 alpha during early development [12]. Expression was induced by exposing embryos to an incubation temperature of 34.5 °C for 18 hours, which resulted in 100% infertile males (presumably a mix of genetic males and females). Although effective, this inducible system would be expensive to apply commercially, the production of all-male stocks is undesirable for some species (including most salmonids and cyprinids), and still has the risk of escapement of fertile transgenic broodstock. This system is similar in risk to triploidy, a chromosomal technique that sterilizes fish, it is not 100% effective as not all fish are triploidized, and this technology also requires fertile broodstock capable of producing fertile offspring on escapement.

Repressible transgenic sterilization was first conceived by Thresher and his collaborators for control of invasive common carp in Australia and also proposed for management of other invasive species [13–15]. This technology is based on genetically disrupting gonadal development, formation of gametes, or embryonic development [13,14,16–21], which can be repressibly restored when needed to produce broodstock. In such approaches, even if broodstock escape and breed, in the absence of the repressor element (usually a chemical compound), offspring are infertile or do not develop, and the transgene persists in the wild for a maximum of one generation. Such a system ("sterile feral technology") has been developed on the basis of a modified tet-off system, in which an early embryonic promoter is coupled to a repressible element that in turn drives expression of a blocker gene, antisense RNA, double-stranded RNA (dsRNA), sense RNA, or ribozyme targeted to a key early developmental gene [14,18]. The repressible element, doxycycline (dox), is administered to water during incubation of embryos. The sterile feral system has been demonstrated, with successful knockout and repression/rescue of critical developmental stages in zebrafish, channel catfish, and common carp [18]. The aforementioned tet-off system could have several disadvantages when used in commercial-scale application. The use of dox treatment could lead to environmental issues and be expensive, and the transgene includes bacterial and viral sequences, which may cause concern among regulators and the general public.

Alternative promoter and repressor systems may be necessary to alleviate these concerns [19–21]. Such an alternative strategy would require a promoter that is sensitive to a compound that is not usually found in significant quantities in the freshwater environment, which would not harm the developing embryos, and would be effective when used in small economical quantities to repress the promoter. A candidate compound that is commonly used in catfish culture is copper sulfate, which is widely used to treat fungus in hatcheries [22]. The copper (Cu) concentration lethal to channel catfish juveniles was 1.75 ppm Cu (6.89 mg/L of $CuSO_4$) [23]. Copper concentration averages 0.004 ppm in surface water [24]. At the initiation of this experiment, we found that continual exposure of channel catfish embryos to 0.1-ppm copper sulfate did not affect embryonic health and survival. Thus, a copper-sensitive promoter was needed, which can be repressed at 0.1 ppm, but actively expresses at 0.01 ppm or less. A candidate promoter system that could meet these requirements is from the yeast (*Saccharomyces cerevisiae*) copper transport protein gene, *CTR3*. *CTR3* is copper sensitive, repressed by elevated copper levels, and regulated by the copper-sensing transcription factor *MAC1* (essential for downregulation or upregulation of the copper transport genes) [25]. It is also strongly downregulated by aqueous copper, with the additional benefit that removal of sequences from the *CTR3* promoter has resulted in promoters of variable sensitivity to copper [25].

Logical target genes to knockdown to sterilize fish would include those associated with primordial germ cell (PGC) migration. Primordial germ cells give rise to gametes that are necessary for fertilization of an organism of the next generation. Primordial germ cell–related genes, such as *nanos*, *vasa*, and *dead end* (*dnd*), are responsible for PGC migration, colonization of the genital ridge, and ultimately gamete formation in fishes. The zebrafish *nanos* gene is essential for PGC formation, proper migration and survival [26]. Primordial germ cells still formed when the *nanos1* expression level was reduced, but they migrated abnormally; the PGCs migration stopped, and they eventually died when *nanos* was knocked out, illustrating that total knockdown of expression may not be needed to disrupt