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## MASS PRODUCTION OF STERILE FISH: HOW CAN WE PRODUCE GAMETES FROM STERILE FISH?

The escape of captive fish from net cages is a serious global problem, as some of the farmed fish are highly domesticated and their genetic background differs from that of their wild counterparts. While the adoption of sterile fish seeds is desirable for avoiding genetic pollution, the mass production of sterile seeds is difficult. Using triploid fish is not considered ideal as some triploids are capable of producing aneuploid gametes. Mature triploids can also engage in mating behavior, potentially ruining the gametes of their wild counterparts.

We employed medaka that were mutants for follicle-stimulating hormone receptor (FSHR), produced by targeting induced local lesions in genomes (TILLING), as the female homozygous mutants are completely sterile. Since the homozygous males are fertile, production of sterile seeds can be achieved by mating heterozygous females with homozygous XX sex-reversed males. However, only half of the resulting F1 individuals are homozygous, and identifying which of these F1 individuals were sterile homozygous mutants would require that they be subjected to DNA analysis before they could be used on fish farms, which would be impractical. To overcome this problem, we applied germ cell transplantation. Since FSHRs are expressed only in gonadal somatic cells and not in germ cells, fertile surrogate females could potentially be produced by transplanting germ cells isolated from homozygous mutants for FSHR into wild-type recipients. These surrogate females would then nurse the mutant germ cells to maturity in a normal gonadal environment and in the presence of wild-type FSHR.

To assess the potential viability of this strategy, we transplanted XY spermatogonia isolated from mutants homozygous for FSHR into sterile triploid female recipients. The resulting surrogate females were then mated with homozygous mutant males to confirm if they were capable of producing only homozygous mutants in the F1 generation. The results showed that fertility was recovered in the triploid female recipients that received homozygous mutant spermatogonia, and also that these recipients produced donor-spermatogonia-derived eggs. By mating the resulting female recipients with homozygous mutant males, fertilized eggs exhibiting normal developmental ability were obtained. Analysis of the FSHR locus in these progeny by CEL1 nuclease and direct sequencing confirmed that all of the F1 larvae were homozygous mutants for FSHR. Further, genotyping of these F1 larvae using male-specific DMY primers revealed that nearly one quarter were females. Thus, we produced female medaka recipients that produced only FSHR-mutant eggs.

We successfully demonstrated that by transplanting germ cells of FSHR homozygous mutant medaka into triploid female recipients, surrogate females that produce only FSHR-mutant eggs can be generated. As a next step, germ cells derived from XX sex-reversed males will be transplanted into triploid female recipients, which will then be mated with XX sex-reversed homozygous mutant males in order to ensure total sterility of the fish seeds in the next generation. By combining genome editing methods such as Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) and the techniques developed in this study, complete sterilization of commercially important species can be achieved.