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Article *in* AACL Bioflux · June 2017

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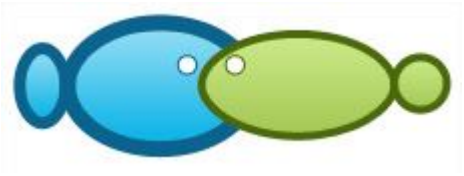

Generation of transparent goldfish

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Abstract. A transparent goldfish strain that allows researchers to study the morphology and distribution of its cells and tissues *in vivo* was established. The strain is considered to be especially useful for visualizing the dynamic processes of cell proliferation and juvenile gonadal development in the goldfish *in vivo*. A relatively transparent variant of the goldfish, Muse, which lacks iridophores, was crossed with an N-ethyl-N-nitrosourea-mutagenized normal goldfish to establish the transparent strain. The strain is highly transparent, and the organs inside body are easily observed in the living fish.
Key Words: transparent goldfish, Muse, Wakin, ENU.

Introduction. Much progress has been made in clarifying the molecular events in the meiotic maturation of the fish oocyte. The goldfish, *Carassius auratus* is one of the most extensively studied fish species and provides a well-characterized model of hormonal regulation and cell-cycle regulation (Nagahama & Yamashita 2008). The molecules that regulate the cell cycle, such as the cdks, cyclins, and c-mos, have been identified and their roles analyzed (Yamashita et al 1992; Katsu et al 1995). The enzymes in the ubiquitin-proteasome pathway have also been identified and characterized (Tokumoto 1999). The receptors for meiotic-maturation-inducing hormones (MIHs), the membrane progestin receptors, have been identified and their roles in the induction of oocyte maturation investigated (Zhu et al 2003; Tokumoto et al 2006). The study of fish oocyte maturation has made an important contribution to our understanding of the mechanisms of action of steroid hormones. During oocyte maturation, the steroid hormone MIH acts on the cell surface through its membrane receptor. Therefore, it is thought that the main pathways by which oocyte maturation is induced are activated by MIH through a nongenomic pathway, in which posttranslational modifications activate the enzymes or factors responsible for oocyte maturation. Therefore, an *in vitro* assay of oocyte maturation in fish is a good model in which to study the mechanism underlying the nongenomic actions of steroid hormones (Tokumoto 2014). Researchers use more than 20 oocytes for each *in vitro* assay of oocyte maturation. Therefore, it is necessary to prepare large numbers of oocytes for these experiments, including the evaluation of the effects of chemicals on the meiotic cell cycle. The goldfish offers advantages in this context because it produces large numbers of oocytes per fish, and several thousand oocytes are generally obtained from a single female. Using *in vitro* assays with goldfish oocytes, we identified the agonistic activity of diethylstilbestrol (DES) on the induction of maturation (Tokumoto et al 2004). DES, a nonsteroidal substance, is a known endocrine disruptor and its effects have been extensively studied. The potent inhibitory effect of pentachlorophenol, one of the most extensively used pesticide throughout the world, on the oocyte maturation induced by MIH and DES has also been demonstrated (Tokumoto et al 2005). For the *in vitro* oocyte maturation assay, the ovary must be excised from the goldfish. However, the bodies of goldfish are naturally opaque, and their ovaries are not

externally visible, so it was difficult to determine the best time for using female goldfish in this research. Therefore, we undertook to produce a transparent goldfish.

Transparent model fish have recently been established in the medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), and guppy (*Poecilia reticulata*). The transparent medaka, also referred to as the "see-through medaka" strain, was established by crossing four natural color mutants (Wakamatsu et al 2001). In the see-through strain, the pigments in the melanophores, xantho-erythrophores, certain types of iridophores, and leucophores were genetically lost from the whole body. See-through zebrafish strains, *roy* and *ruby*, were also established and have advantages in bioimaging (White et al 2008). Recently, we established ovarian-fluorescent transparent zebrafish strains *β-roy* and *β-ruby* (Akhter et al 2016). Using these strains, we successfully performed a sex-change experiment, exploiting their transparency to observe the changes in the ovaries inside the body (Takatsu et al 2013). The *See-Thru-Gonad* zebrafish line was also established by closing transgenic line of the germ-cell-specific expressing gene, *vasa*, and transparent strain (Presslauer et al 2016). This strain allows the observation of the gonad throughout the whole life of the fish. A "see-thru guppy" strain has also been established (Shaddock 2009) because the guppy is an attractive model for the study of evolutionary ecology.

Although there have been no scientific reports of transparent goldfish, a transparent goldfish was reported on a Web site by Dr. Y. Tamaru, Mie University. However, the transparency of that goldfish is similar to the transparency of Muse, and the organs in abdomen cannot be seen in the picture. Therefore, we attempted to produce a fully transparent goldfish. Various variants of the goldfish have been developed during its long history as a pet animal. One of these goldfish variants, Muse, seems to have lost most of its iridophores, but is opaque, with a white body color. In this study, we tried to improve the transparency of Muse with mutagenesis, using N-ethyl-N-nitrosourea (ENU).

Material and Method

Materials. The original strains of goldfish used, Muse and Wakin, were purchased from a local supplier (Figure 1). ENU was dissolved to a concentration of 100 mM (1000-fold stock) in 5 mM 2-morpholinoethanesulfonic acid (MES; pH 7.0) and stored at -20°C until use.

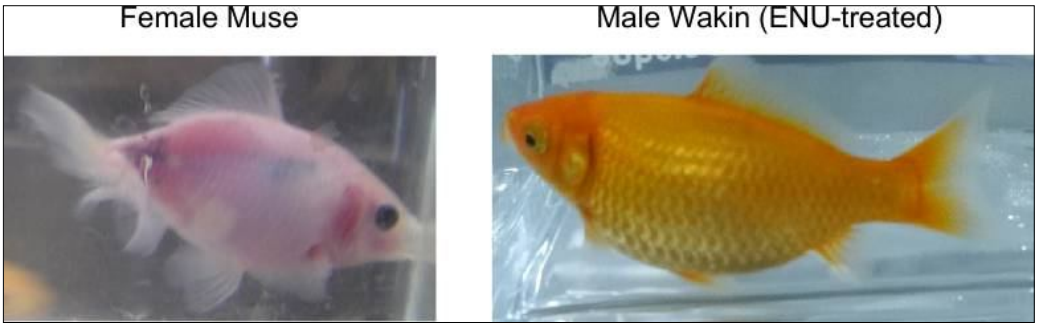

Figure 1. Original female and male goldfish (*Carassius auratus*).

Keeping goldfish. A group of adult goldfish of 20-50 male and female was kept in 150 cm x 60 cm square and 90 cm high acryl case with continuous circulating water through filtration sands. Aquarium was filled with charcoal-filtered tap water (pH 6.5 to 7.4, 14-20°C). The fish were fed dried pellet twice a day. The maintenance of aquarium, changing water and clean the sands, was conducted once in two months.

Paring of goldfish. The subsequent generations of fish were obtained by natural mating. Pairings were conducted after 12 months old. Sexually matured female and male goldfish were selected from a keeping aquarium described above and separated into each fish in 20 cm x 20 cm square and 25 cm high glass case. During spawning season

(January to May in our fish keeping room), goldfish were kept separately at 20 to 24°C and used for paring two to three weeks interval. Each mating pair was housed in 60 cm x 30 cm square and 45 cm high glass case with continuously bubbled up with air pump and set with spawning floor prepared with Lily of the Valley tape for several days until spawning. After spawning, parent fishes were removed and eggs were leaved in case. After about one week, feeding for juveniles was started. Juveniles were fed paramecium and dried brine shrimp egg powder. Juveniles were kept in same case until they were one month old. Then, juveniles were separated into other cases according to fish number.

Results and Discussion. A male Wakin fish was injected with human chorionic gonadotropin (250 IU) for ejaculation. On the following day, the male fish was treated with 0.1 mM ENU *in vivo* by adding ENU to the water at room temperature for 15 h. After the male fish was treated three times with ENU at 1 week intervals, the female Muse and ENU-treated Wakin fish were placed in an aquarium to mate. The eggs were collected after natural spawning. F1 juveniles were obtained from the cross between the female Muse with the ENU-mutated male Wakin fish and maintained in a closed colony (Figure 2).

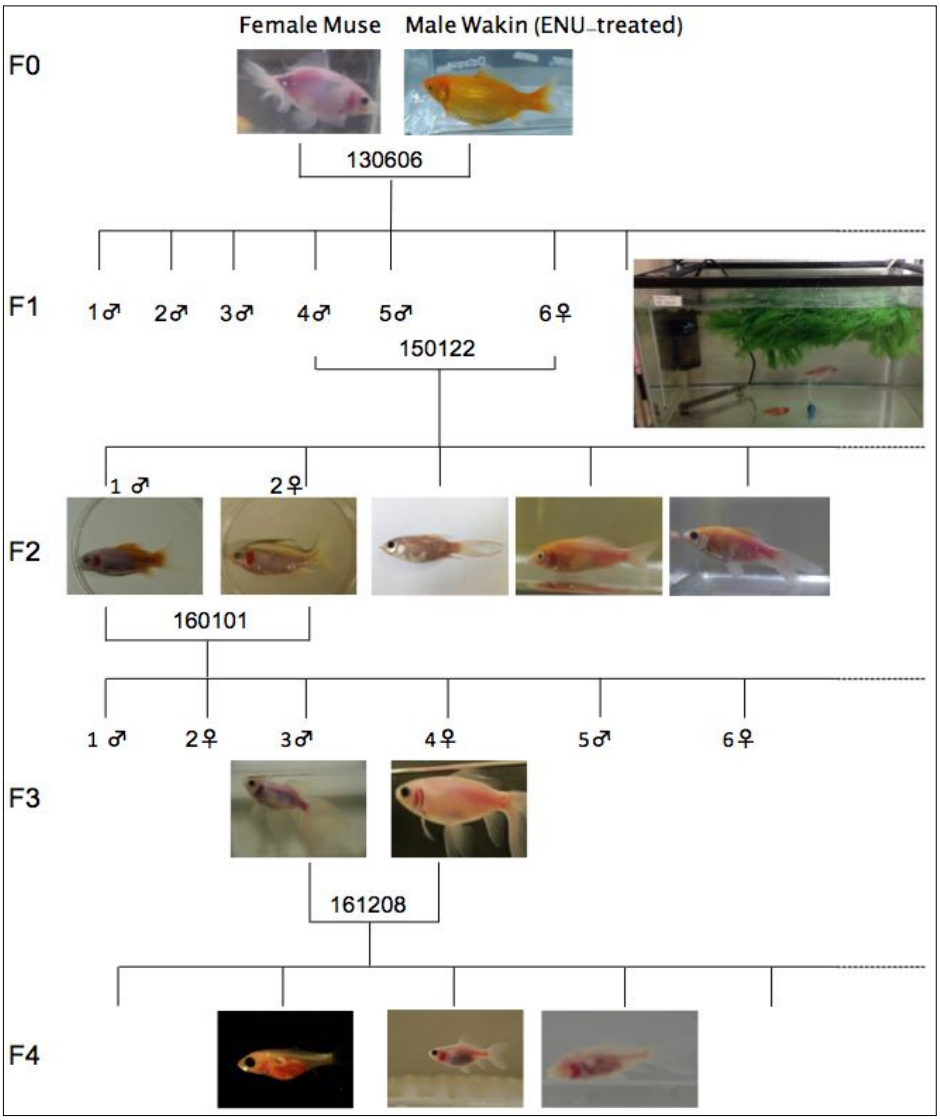

Figure 2. Schematic representation of the development of the transparent goldfish line. The date upon which we first paired the F0 generation was 6th June 2013, indicated as 130606. The dates of pairing in the later generations are indicated in the same way.

We selected several juveniles and intercrossed them twice (F1-F3 generations). The phenotype of Muse was not recessive because about half the juveniles showed the Muse phenotype. However, the phenotype showed high mosaicism. Some silver scales remained on parts of the fish bodies. We then selected fish with no silver scales to produce the next generation. After selection for two generations, we produced several constantly transparent goldfish in the F3 generation (Figure 3).

Female

Male

Figure 3. Female and male transparent goldfish produced in the F3 generation.

By crossing these fish, we successfully produced an F4 generation in which 100% of the fish had transparent bodies (Figure 2, Figure 4A and B). The juveniles are highly transparent until they are several months old, and the organs inside the body are visible (Figure 4B). The body becomes slightly white by glowing, but is still transparent until 1 year old, and the gonads are visible from outside the body (Figure 4C).

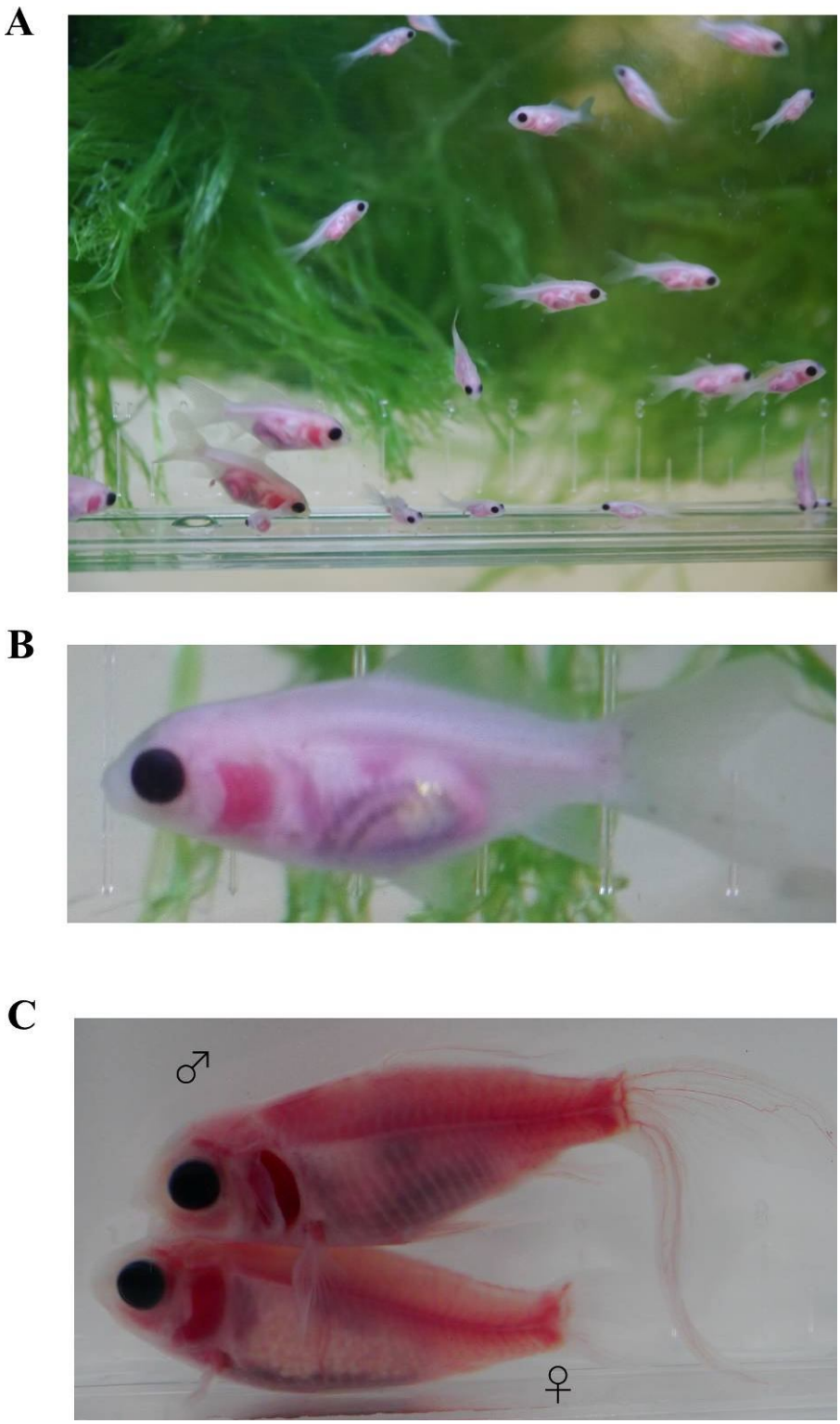

Figure 4. Transparent goldfish produced in the F4 generation: (A) juvenile transparent goldfish; (B) enlarged image of a juvenile; (C) one-year-old male and female goldfish.

Fish have four main types of pigment cells, melanophores, iridophores, leucophores, and xanthophores (Fujii 2000). The main function of these iridophores is to make the fish body opaque. Iridophores display various structural colors and iridescence by reflecting light from the surfaces of evenly distributed organelles reflecting platelets (Presslauer et al 2016). The transparent mutant zebrafish phenotype is derived from the loss of iridophores (Kraus et al 2013). Therefore, we infer that the strain established in the present study has a defect in the formation of iridophores. However, the fish body

gradually became slightly white. The white color of fish is attributable to leucophores, which are considered an extreme type of iridophore. Therefore, the ability to form leucophores is still retained in the transparent goldfish established in this study. Consequently, it may be possible that improve the transparency of these transparent goldfish. Although we cannot explain the improvement in transparency caused by the ENU treatment, we know that ENU induced mutations in these fish because we also obtained eyeless mutants in the F4 generation (Figure 5). However, it is difficult to identify the affected genes in the genome, although the genes mutated to produce transparency may be identified in future research.

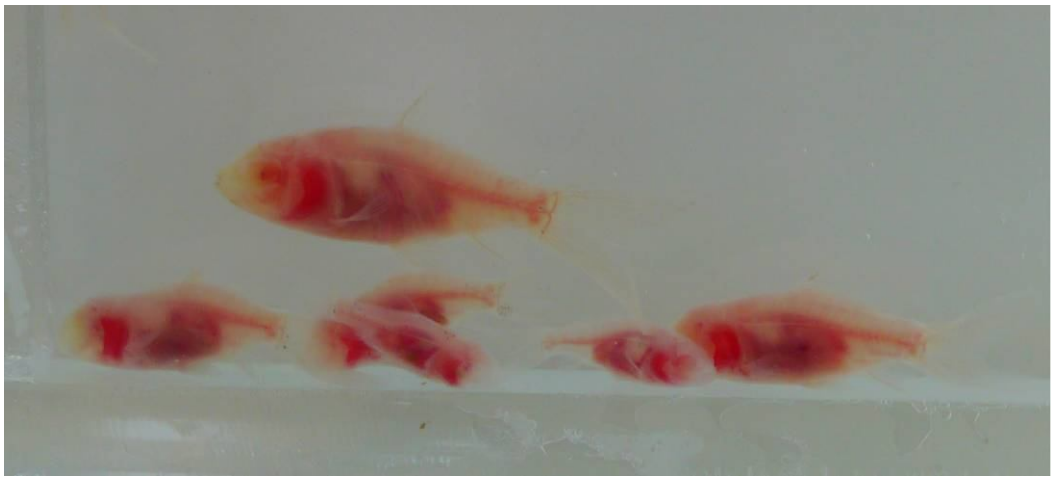

Figure 5. Eyeless mutants of the transparent goldfish produced in the F4 generation.

Conclusions. We have established a highly transparent goldfish strain. The strain is fertile and can therefore be used as an experimental model. The transparent goldfish will allow researchers to study the development of tissues and organs in live fish. The transparent fish is also an ideal model organism in which to evaluate the effect of chemicals on organ development.

Acknowledgements. This work was supported by Grants-in-Aid for Scientific Research in Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank M. Tokumoto and T. Tokumoto for the maintenance of the goldfish.

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Received: 12 May 2017. Accepted: 07 June 2017. Published online: 24 June 2017.
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How to cite this article:
Wang J., Klangnurak W., Naser A. A., Tokumoto T., 2017 Generation of transparent goldfish. AACL Bioflux 10(3):615-621.