Insertional mutagenesis in *ChordinA* induced by endogenous $\Delta Tgf2$ transposon leads to bifurcation of axial skeletal systems in grass goldfish

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The grass goldfish appeared early in the evolutionary history of goldfish, and shows heritable stability in the development of the caudal fin. The twin-tail phenotype is extremely rare, however, some twin-tail individuals were produced in the process of breeding for ornamental value. From mutations in the twin-tail goldfish genome, we identified two kinds of $Tgf2$ transposons. One type was completely sequenced $Tgf2$ and the other type was $\Delta Tgf2$, which had 858 bp missing. We speculate that the bifurcation of the axial skeletal system in goldfish may be caused by an endogenous $\Delta Tgf2$ insertion mutation in *ChordinA*, as $\Delta Tgf2$ has no transposition activity and blocks the expression of *ChordinA*. The twin-tail showed doubled caudal fin and accumulation of red blood cells in the tail. In addition, *in situ* hybridization revealed that ventral embryonic tissue markers (*eve1*, *sizzled*, and *bmp4*) were more widely and strongly expressed in the twin-tail than in the wild-type embryos during the gastrula stage, and *bmp4* showed bifurcated expression patterns in the posterior region of the twin-tail embryos. These results provide new insights into the artificial breeding of genetically stable twin-tail grass goldfish families.

Goldfish (*Carassius auratus*) are one of the three famous ornamental fish, and are known as the “water fairy.” Goldfish evolved from wild crucian carp and mutated into different species (grass goldfish, wen goldfish, dragon-eye goldfish, and oval goldfish). Each species has unique characteristics, but the most characteristic phenotype is the bifurcated caudal fin\(^1\). The caudal fin of aquatic animals is mainly divided into two types (wild-type and twin-tail) and goldfish are representative species of the twin-tail. The goldfish tail is supported by the caudal axial skeleton, but the twin-tail has bifurcated fin folds that differ from the wild-type. There are many factors that caused bifurcation of the goldfish caudal fin, such as growth environment, genetic factors, and epigenetic effects. Previous reports showed that the development of the goldfish caudal fin is closely related with regulatory factors in the ooplasm\(^2,3\). In addition, a single nucleotide mutation at the 127\(^{th}\) amino acid base in *ChordinA* restricted its transcription regulation, which generated the twin-tail phenotype\(^4\).

*Tgf2*, the second transposon in vertebrates, belongs to the hAT family and is also found in goldfish. *Tgf2* plays an important role in transgenesis and gene trapping with independent transposition activity\(^5,6\). Previous research on transgenesis was carried out by coinjecting the vector plasmid and transposase mRNA into zebrafish, grass carp, and goldfish. The results showed that the eGFP reporter gene was transferred from the vector plasmid into grass carp and goldfish genomes, with an incorporation efficiency of 96% and 37%, respectively. In addition, the *Tgf2* transposon in goldfish has three other copies that belong to the goldfish *Tgf2*-Ds group ($\Delta Tgf2$) and have no independent transposition capability\(^5,7,8\).

In this study, we obtained the twin-tail phenotype induced by the endogenous $\Delta Tgf2$ transposon insertional mutation and conducted pedigree breeding to obtain a genetically stable twin-tail goldfish strain. We also conducted functional assays and analyzed the expression patterns in ventral embryonic tissue markers.
Results

The discrimination of wild-type and twin-tail goldfish. In the 10 families (Tgf2 family-1–4 and ΔTgf2 family-1–6), all the goldfish from the Tgf2 family-1 and -2 were wild-type, more than 95% of goldfish from family-3 and -4 were wild-type, and only a few individuals were twin-tailed (Fig. 1a). Similarly, all the goldfish from ΔTgf2 family-1 and -3 were wild-type, more than 92% of goldfish from family-4 and -5 were wild-type, and only a few were twin-tail (Fig. 1d). In contrast, 89% and 100% goldfish from ΔTgf2 family-2 and -6 were twin-tailed, respectively. As such, we inferred that endogenous ΔTgf2 may participate in the generation of, but not necessarily induce, the twin-tail phenotype.

To examine how the endogenous ΔTgf2 contributed to the twin-tail phenotype, we amplified and sequenced cDNA from wild-type and twin-tail goldfish. In the twin-tail goldfish, ΔTgf2 was found inserted into 401 nt

Figure 1. Test of the insertion site of the ΔTgf2 transposon in grass goldfish. Different families with or without the Tgf2 transposon were established to analyze the frequency of wild-type and twin-tail grass goldfish, respectively. (a,d) Observed Tgf2 and ΔTgf2 transposon insertion copies (b,e), and the position of ChordinA insertion (c,f).
in the 2nd exon of ChordinA. We named the gene as ChordinAΔTgf2 and described the homozygous genotype as ChordinAΔTgf2/ΔTgf2 (Fig. 1f). Conversely, in the wild-type goldfish, no insert was found in ChordinA and we named the gene ChordinA+, and described the homozygous genotype as ChordinA+/+ (Fig. 1c). A previous report showed that ChordinA plays an important role in the differentiation of the caudal fin in goldfish4, and we speculate that the insertion of endogenous ΔTgf2 leads to mutagenesis in ChordinA, which results in bifurcation of the caudal fin.

Breeding of twin-tail goldfish strains based on the ΔTgf2 transposon. When ChordinA+/+ individuals were crossed with ChordinAΔTgf2/ΔTgf2, the F1 group (~375 individuals) were vent group (ChordinA+/ΔTgf2), and the F2 group (~846 individuals) was separated into three types: wild-type (wt) larvae from wild-type (chordinA+/+) embryos (~589 individuals), single caudal fin (vent) larvae from weakly-ventralized (chordinA+/ΔTgf2) or bifurcated caudal fin fold (chordinAΔTgf2/ΔTgf2) embryos (~21 individuals), and bifurcated caudal fin (twin) larvae from bifurcated fin fold (chordinAΔTgf2/ΔTgf2) embryos (~236 individuals) (Fig. 2a, Table 1). At 2 dpf, the wt phenotype showed the single tail phenotype. The vent phenotype with the incomplete forked fin folds was characterized by a single tail and the twin phenotype with completely divided fin folds showed twin-tail phenotype. However, the vent and twin individuals have a common characteristic that they have the accumulation of red blood cells in the tail (Fig. 2b). The F3 group, which included 317 twin individuals (93%) and 23 vent individuals (7%), suggested that the vent phenotype may be atavism due to the external environment and/or other genetic factors9,10.

Comparison between wild-type and twin-tail goldfish. We compared embryonic and adult fish to distinguish between wild-type and twin-tail goldfish. We observed several different characteristics between the two strains of goldfish. Morphologically, wild-type goldfish share characteristics with wild-type crucian carp.
Carassius auratus) and have a traditional dovetail-shape tail lobe (Fig. 3a). Meanwhile, in the evolutionary history of the grass goldfish, a twin-tail phenotype appeared with bifurcated fin folds. Alizarin red staining of axial skeletons is a practical method to analyze the anatomical structure of goldfish tails. Caudal axial skeletal elements were identified according to previous reports. The results showed that the tail nerve bone of both wild-type and twin-tail goldfish were completely mineralized and formed the caudal fin. The caudal fin were attached to the Ep (epural bone), Hy (hypural bone), and Ph (paranasal bone of the hypural bone), however, the quantification data of Nc (notochord), Ph, and Hy1~6 in the wild-type and twin-tail goldfish showed no obvious difference (Table 2). The number of caudal fin rays in wild-type goldfish was 20 to 24, arranged in a double-leaf type shape (Fig. 3b,c), while that of twin-tail goldfish was 39 to 47, arranged in a four-leaf type shape (Fig. 3f,g). Histologically, the characteristics of the axial skeleton demonstrated that the origin of the twin-tail phenotype was attributable to double germination of Hy and Ph (Fig. 3h), which were developed non-redundantly in the wild-type goldfish (Fig. 3d). Embryological results showed that accumulation of red blood cells in bifurcated fin folds only occurred in twin-tail goldfish, and not in wild-type goldfish (Figs 2b and 4). This was consistent with previous reports.4

Table 1. Phenotypes and genotypes of goldfish strains.

| Group | Genotype | Phenotype |
|-------|----------|-----------|
|       |          | Wt | Vent | Twin |
| F1    | chordinA\( ^{+/-}\) | 375 | 0 | 0 |
| F2    | chordinA\(^{++}\) chordinA\( ^{+/+}\) | 589 | 21 | 236 |
| F3    | chordinA\( ^{+/-}\) | 0 | 23 | 317 |

Table 2. The quantification data of nc, ph, and hy1~6 in the Wt and Twin.

| Group | Wt | Twin |
|-------|----|------|
| Characteristic | Width (cm) | Length (cm) | Width (cm) | Length (cm) |
| nc     | 0.037 | 0.028 | 0.058 | 0.058 |
| hy6    | 0.037 | 0.05  | 0.026 | 0.073 |
| hy5    | 0.091 | 0.15  | 0.039 | 0.11 |
| hy4    | 0.05  | 0.106 | 0.036 | 0.125 |
| hy3    | 0.031 | 0.072 | 0.054 | 0.048 |
| hy2    | 0.037 | 0.059 | 0.054 | 0.075 |
| hy1    | 0.05  | 0.209 | 0.048 | 0.221 |
| ph     | 0.066 | 0.269 | 0.048 | 0.188 |

Figure 3. Tail morphology of grass goldfish. Different observation methods were used to obtain the external morphology (a,e), the skeletal structure (b,f,c,g), and the transverse section of the caudal fin. (d,h) nc (notochord), hy (hypural bone), ph (paranasal bone of the hypural bone), hs (haemal spine).
axis and can control the caudal fin phenotype of goldfish. On the contrary, during the gastrula stage, expression patterns were different between wild-type and twin-tail embryos. During the gastrula stage (80% epiboly), ChordinA is a marker gene, mainly expressed in the hindbrain, which is usually used as a positive control for other ventral marker genes in the DV axis. Bmps also function in skeletal development and the dorsal-ventral axis of vertebrate embryos is thought to be specified by a gradient of bone morphogenetic protein (bmp) activity. To determine how the insertional mutation in ChordinA that was mediated by endogenous Tgf2 contributed to DV patterning in twin-tail goldfish embryos, we examined the expression patterns of ChordinA, ventral embryonic tissue markers (eve1, sizzled, and bmp4), and a hindbrain marker (krox20) in the embryos of wild-type and twin-tail goldfish. The results showed that the expression patterns were different between wild-type and twin-tail embryos. During the gastrula stage (80% epiboly), ChordinA was expressed in the outwards germ layer and showed wider expression patterns in wild-type (Fig. 6a–c) than in twin-tail embryos (Fig. 6d–e). Similarly, during the segmentation stage, ChordinA was mainly expressed in the tailbud and showed obviously wider expression patterns in wild-type (Fig. 6d,e) than in twin-tail embryos (Fig. 6d,e). A few reports have suggested that ChordinA plays an important role in the development of the DV axis and can control the caudal fin phenotype of goldfish. On the contrary, during the gastrula stage, eve1 and sizzled showed stronger signal and wider expression patterns in twin-tail (Fig. 6f–h) than in wild-type embryos (Fig. 6f–h) in the posterior-ventral regions. bmp4 is one of the important determining factors for ventral embryonic development. During the late gastrula stage (40% epiboly), the expression of bmp4 was stronger in twin-tail than in wild-type embryos (Fig. 6i). In addition, bmp4 expression patterns in wild-type and twin-tail embryos showed obvious differences during the segmentation stage. As shown in Fig. 6k,l, bmp4 showed bifurcated expression patterns in the posterior region of twin-tail embryos, which were not observed in the wild-type embryos (Fig. 6k,l). This indicated that the axial skeleton became bifurcated and doubled during the segmentation stage in the twin-tail goldfish. The ventral embryonic tissue marker expression patterns were consistent with a previous study of dino zebrafish mutants and other chordin-deficient embryos. The results showed that krox20 expression patterns were not different between wild-type and twin-tail embryos, which differed from previous reports of significantly reduced in krox20 expression patterns in chordin-deficient vertebrate embryos. This indicated that the formation of the DV axis played an important regulatory role in the differentiation of tail-patterns in goldfish.

Discussion
The generation of diversified caudal fins in goldfish is caused by a mutation. Previous studies have found that Chordin mutants generate bifurcated caudal axial skeletons. This natural mutation has been produced and inherited consistently through artificial selection since the song dynasty of China. The Tgf2 transposon has not been observed in wild crucian carp, but occurs in grass goldfish as well as other goldfish strains. In the grass goldfish genome, the complete sequence type (Tgf2) and the multiple missing pieces type (ΔTgf2) have been detected, which indirectly indicated that grass goldfish is the ancestor of many other strains. The Tgf2 transposon relies on its encoded transposase to mediate translocation. Under the artificial selection, those goldfish whose morphological variation caused by transposon were preserved, this may be

![Figure 4](image-url) Embryonic observations during the incubation period of the wild-type and twin-tail grass goldfish. Arrows point to the abdominal stem where a large number of red blood cells accumulated in twin-tail grass goldfish.
because the Tgf2 transposon retained their independent transposition activity through long-term evolution. Generally, there are large differences between goldfish parents and progeny in terms of color and proportions, which could be due to the insertion mutation of the Tgf2 transposon. However, the ΔTgf2 transposon are unable to produce transposase and has no independent transposition capability. According to Mendel’s law, ΔTgf2 homozygote will be generated when the goldfish with ΔTgf2 was selfed. Therefore, the phenotype caused by the ΔTgf2 insertion mutation might be genetically stable. A limited number of individuals with the mutation might have survived. Therefore, we speculated that such a large number of twin-tail goldfish from only 10 families were not directly produced by the insertion mutation, but from several generations of hybridization of insertion mutation individuals. In previous studies, in situ hybridization of Tgf2 showed that it has different expression patterns in different embryos and was detected in the head and tail. In our study, Tgf2 can be detected in the tail. Our result is consistent with previous reports of Tgf2 expression patterns. This may be caused by the Tgf2 has independent transposition capability. The insertion site in the goldfish genome affects the expression of Tgf2 transposase. However, no transposase mRNA was detected in ΔTgf2 embryos confirming the ΔTgf2 with large deletion lost its independent transposition capability. In this study, ΔTgf2 transposon was found inserted in the 2nd exon of ChordinA in the twin-tail goldfish. And it is precisely because ΔTgf2 has no independent transposition capability, insertional mutagenesis in ChordinA inhibited the expression of the 2nd to 4th exons of ChordinA.

Previous studies showed that a single nucleotide mutation in the 127th amino acid of ChordinA (on the 2nd exon, prior to the ΔTgf2 insertion site) generated a stop codon and produced the twin-tail phenotype, we speculate that
the bifurcation of the axial skeletal system in goldfish may be caused by an endogenous \( \Delta Tgf2 \) insertion mutation in \( ChordinA \). However, the relation between the insertional mutation mediated by \( \Delta Tgf2 \) transposon and a single nucleotide mutation in \( ChordinA \) require further study.

Alizarin red staining showed that the tail nerve bones forming the caudal fin of both wild-type and twin-tail goldfish were completely mineralized. The difference was that the elements of the axial skeleton system of twin-tail goldfish were doubled with bifurcated fin folds. \( In situ \) hybridization indicated that the expression patterns of the two goldfish strains were different during the gastrula stage. The expression of ventral embryonic tissue genes increased significantly due to the \( \Delta Tgf2 \) insertional mutation in \( ChordinA \). This indicated that the ventral tissue had proliferated, which generated the double caudal fin\(^{35}\). In addition, \( ChordinA \) showed wider expression patterns in wild-type goldfish than in twin-tail goldfish embryos, during both the gastrula and segmentation stages. However, the reduced expression of \( ChordinA \) can also lead to the generation of the twin-tail phenotype. Therefore, we speculate that the insertional mutation in \( ChordinA \) mediated by \( \Delta Tgf2 \) contributed to the generation of twin-tail by suppressing the inhibitory effect of \( ChordinA \).

Insertional mutagenesis induced by the \( \Delta Tgf2 \) transposon has mainly been detected in goldfish, so we speculate that this insertional mutation has a special regulatory role in the generation of twin-tail goldfish\(^{34,35} \). Based on this study, we infer that insertional mutagenesis mediated by the \( \Delta Tgf2 \) transposon was the main cause of twin-tail; however, it was also affected by other genetic and/or environment factors. By exploring the mechanism of \( \Delta Tgf2 \) insertional mutagenesis and the generation of the goldfish caudal fin, these results provide a theoretical basis for artificial breeding of genetically stable twin-tail grass goldfish families.

Materials and Methods

Experimental goldfish and embryos. \( P_0 \) generation of wild-type and twin-tail goldfish were obtained from the Genetics and Breeding Center of Shanghai Ocean University, Shanghai, China. Embryos were generated by artificial insemination. Fertilized eggs (~150) were placed in petri dishes (10 cm in diameter) and development at room temperature (22 ± 1 °C). Petri dish with well-aerated water to maintain normal dissolved oxygen (DO) levels during embryogenesis and replaced every 4 hours. Embryos used for RNA extraction were stored by immersion in RNA Store (ABigen, Beijing, China) and kept in 4 °C overnight and then −80 °C until used. Embryos collected for \( in situ \) hybridization analysis were kept in embryonic medium supplemented with 0.003% (w/v) 2-phenylthiourea to prevent pigmentation, then fixed overnight at 4 °C in 4% paraformaldehyde (PFA) and then −20 °C in methyl alcohol. Juvenile goldfish used for anatomical and histological analyses were sacrificed by immersion in MS-222 (tricaine methanesulfonate, Sigma, St. Louis, MO) for 5 min. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Advisory Committee for Laboratory Animal Research and conducted following the guidelines approved by the Shanghai Ocean University Committee on the Use and Care of Animals.

Family construction and breeding of goldfish. In breeding experiments, we observed that the twin-tail phenotype occurred in the wild-type family. From the mutation in the twin-tail goldfish genome, we identified two kinds of \( Tgf2 \) transposon, one that was completely sequenced (\( Tgf2 \)), and one with multiple pieces missing (\( \Delta Tgf2 \)) (Fig. 7). To detect the relationship between the \( Tgf2 \) transposon and the differentiation in the caudal fin, we constructed 10 families (\( Tgf2 \) family-1–4 and \( \Delta Tgf2 \) family-1–6) for analysis. Families were divided based on the results of \( Tgf2 \) sequencing and each family contained 100 fishes. Primer set \( Tgf2-F1/-R1 \) (designed base on accession number HM146132.1, Table 3) was used to amplification \( Tgf2/\Delta Tgf2 \) sequence.

To obtain a genetically stable twin-tail goldfish strain, we crossed a wild-type goldfish (\( ChordinA^++/-, \delta \)) with a mutant twin-tail goldfish (\( ChordinA^{\Delta Tgf2/-\Delta Tgf2}, \varphi \)) to obtain the F1 hybrid \( ChordinA^+/-\Delta Tgf2 \). The F1 hybrid individuals were selfed to obtain F2 offspring. Twin-tail strains (\( ChordinA^{\Delta Tgf2/-\Delta Tgf2} \)) selected from the F2 were selfed to obtain F3 individuals. The pedigree structure is shown in Fig. 2a. The phenotypes of goldfish strains were based

![Figure 7. The sequence alignment of \( Tgf2 \) and \( \Delta Tgf2 \). \( Tgf2 \) was the complete sequence type and \( \Delta Tgf2 \) was the missing pieces type.](Image)
Table 3. Primer sequences used in this study.

| Primers names | Primer sequence (5’-3’)         |
|---------------|---------------------------------|
| chordina-A-F1  | TAAAGCAGACAGATGCGACGGTGTG       |
| chordina-A-R1  | TGCCCTTCCTCAACAGTGATTAAGG       |
| eve1-F         | ATCGCTACAGGGCGAGGGGAGG          |
| eve1-R         | TCCTGAAAGCTGGCAAAGGTGTG         |
| sizzled-F      | ACGCTGACTCAAGCCCGCCTGACC        |
| sizzled-R      | GAACCCTCCTCCAGACAGTGTGGG        |
| bmp4-F         | CCGTCATCTGAAGGATGGGAG           |
| bmp4-R         | GGGTGTTTTCGGCGGTGTATGCC         |
| krox20-F       | ATGAGACGTCTAAAACCTTGGAG         |
| krox20-R       | GGCTTTGGGCGCGGCGGTGTATGCC       |
| Tgf2-F1        | TGTGCCGCTGACATTACTTACCC         |
| Tgf2-R1        | CTTACAAAGGTTGAAAAGCCTC          |
| Tgf2-F2        | CCATCATATACACGAGGTAAA           |
| Tgf2-R2        | CTGCTTGAAGGTTTGTTGATTT          |

Probe preparation and sequencing. The probes of ΔTgf2, Tgf2, chordina, eve1, sizzled, bmp4 and krox20 were prepared by using PCR. Total RNA was isolated from goldfish embryos at 44 hpf using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and subsequently treated with DNase (Promega, Madison, WI, USA) to eliminate contaminating genomic DNA. First-strand cDNA was reverse-transcribed from the total RNA using Reverse Transcriptase M-MLV (TaKaRa, Tokyo, Japan) with oligo-dT primers according to the manufacturer’s instructions. Primer sets Tgf2-F1/-R1, Tgf2-F2/-R2 (designed based on accession number AB874473), eve1-F/-R, sizzled-F/-R, bmp4-F/-R and krox20-F/-R (refer to Gembu Abe) (Table 3) were used to amplification probes sequence. PCR products were gel-purified, ligated into the T/A cloning vector pGEM-T (Promega, Madison, WI, USA) and transformed into Escherichia coli DH5α. Positive clones were examined by PCR and direct sequencing.

Whole-mount in situ hybridization. Fixed goldfish embryos were washed briefly in PBS containing 0.1% Tween-20, then transferred to 100% methanol, and stored at −20 °C for a minimum of 24 h. Whole-mount in situ hybridization using digoxigenin (DIG)-labeled RNA riboprobes was performed as reported previously, with modifications. Embryos were photographed using a Nikon SMZ1500 fluorescence microscope (Tokyo, Japan).

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Author Contributions
Y.W.S., D.D.G. and S.M.Z. wrote the manuscript. S.M.Z. designed the experiments. Y.W.S. conducted pedigree breeding. D.D.G. conducted the embryonic observations during the incubation period. Y.W.S., W.T.C., H.H.G., S.K.D. and J.C. conducted functional assays and analyzed the expression patterns in ventral embryonic tissue markers. All authors contributed to finalizing and approving the manuscript.

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