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Letter to the editor Open Access

# Involvement of Sox9a in chondrogenesis and gonadal development in teleost Nile tilapia (*Oreochromis niloticus*)

**DEAR EDITOR,**

Sox9 is a member of the Sry-related high-mobility group box (Sox) transcription factor family in animals. In teleost fish, *Sox9* undergoes duplication to generate two duplicates, namely *Sox9a* and *Sox9b*. However, the functions of these duplicates in the teleost Nile tilapia (*Oreochromis niloticus*) remain unclear. In this study, we characterized the roles of Nile tilapia *Sox9a* in chondrogenesis and gonadal development. *In situ* hybridization assays showed that *Sox9a* was mainly expressed in cartilage tissues and somatic cells surrounding germ cells of the gonads. CRISPR/Cas9-mediated homozygous mutation of the *Sox9a* gene resulted in craniofacial deformities and missed mandibles, as well as impaired the expression of *Col2a1a* that is involved in chondrogenesis. In addition, germ cell number and DNA replication in somatic cells in the gonads of both sexes were reduced following *Sox9a* mutation. Taken together, this study demonstrates that Sox9a is involved in cartilage development and germ cell proliferation in Nile tilapia.

The Sox transcription factor family, found solely in metazoans, is characterized by the presence of a highly-conserved high-mobility group (HMG) box domain. Teleost fish have undergone three rounds of whole-genome duplication, resulting in the proliferation of duplicated genes. In a previous study, we identified 27 *Sox* genes in the genome of the Nile tilapia (Wei et al., 2016). Notably, compared with the Sox family in mice and humans, eight *Sox* genes have experienced duplication and formed two copies in Nile tilapia (Wei et al., 2016). Interestingly, *Sox9* duplication, which generated *Sox9a* and *Sox9b* duplicates, is not only observed in Nile tilapia but also in other teleosts, including zebrafish and medaka.

Increasing evidence has shown that different Sox family members play essential roles in diverse biological processes (Angelozzi & Lefebvre, 2019), including sex determination, testis development, gametogenesis, neural development, skeletogenesis, and cancer progression. In mammals, Sox9 is involved in the regulation of male sex determination and testis development, chondrogenesis, mammary gland development, and biliary development (Angelozzi & Lefebvre, 2019). In teleosts, *Sox9* duplicates exhibit overlapping and distinct expression patterns and functions (Cresko et al., 2003). For example, in zebrafish, *Sox9* duplicates show similar expression levels in the eyes and brain of adults, but divergent expression in the gonads, with dominant expression of *Sox9a* in the testis and *Sox9b* in the ovary (Cresko et al., 2003). Functionally, *Sox9* duplicates in zebrafish are also involved in bone development and chondrocyte morphogenesis, and Sox9b is associated with pigment cell development (Yan et al., 2005). In a previous study, we found that *Sox9a* and *Sox9b* are highly expressed in both the ovary and testis of Nile tilapia at 5 and 30 days after hatching (dah); *Sox9b* exhibits testis-biased expression at 120 and 180 dah; both duplicates are similarly expressed in several adult tissues, including the brain and heart (Wei et al., 2016). However, the functions of *Sox9* duplicates in Nile tilapia remain largely unknown.

In the present study, we investigated the detailed expression and functions of Nile tilapia *Sox9a*. First, we conducted *in situ* hybridization experiments to profile the detailed spatiotemporal expression of Nile tilapia *Sox9a*. Whole-mount *in situ* hybridization showed that *Sox9a* was highly expressed in certain types of cartilage at 3 dah, including craniofacial, pectoral fin, vertebral, and caudal bud cartilage (Supplementary Figure S1). In addition, fluorescence *in situ* hybridization of Nile tilapia gonads detected *Sox9a* mRNA in somatic cells surrounding germ cells of the ovary and testis from 5 to 30 dah, which was undetectable at 60 dah (Supplementary Figure S2). Our data suggest that Sox9a in Nile tilapia may be associated with cartilage and gonadal development during the juvenile stage.

To determine the functions of Sox9a during development, we used CRISPR/Cas9 gene editing to mutate *Sox9a* in Nile tilapia. Guide RNA (gRNA) was designed to target the first exon upstream of the sequences encoding the HMG box domain of *Sox9a* (Supplementary Figure S3A), then microinjected with Cas9 mRNAs into the zygotes. Polymerase chain reaction (PCR) amplification of genomic DNA from F0 fish and subsequent sequencing analysis detected five independent genomic deletions around the target sites within *Sox9a* in the positive mutants compared to wild-type (WT) fish (Supplementary Figure S3B). We next used F1 heterozygous mutants with a 7 bp deletion to establish homozygous *Sox9a* mutants ($Sox9a^{-/-}$). PCR amplification followed by a heteroduplex gel motility assay revealed that PCR products from the heterozygous *Sox9a* mutants ($Sox9a^{+/-}$) contained both heteroduplex and homoduplex bands, while $Sox9a^{+/+}$ and $Sox9a^{-/-}$ only contained homoduplex bands (Supplementary



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Figure S3C). The homozygous *Sox9a* mutation with a 7 bp deletion was further confirmed by sequencing analysis (Supplementary Figure S3D), leading to a frameshift and premature translation termination, thus forming a truncated protein lacking the HMG box domain (Supplementary Figure S3E).

We next analyzed the effects of the homozygous *Sox9a* mutation on Nile tilapia development. Our findings revealed that Nile tilapia fish bearing the *Sox9a* mutation died at 20 dah, likely the result of craniofacial development defects. Notably, morphological analysis indicated that, compared to the WT group, the *Sox9a* mutant group exhibited craniofacial deformities and missing mandibles (Figure 1A, B), which might cause potential feeding impairment. Alcian blue staining revealed that at 10 dah, the homozygous *Sox9a* mutants showed an absence of Meckel's cartilage (m), rostral cartilage (rc), and basisphenoids (bs), displayed hypoplastic lamina orbitonasalis (lon) and taeniae marginals anterior (tma), and showed a shift in the position of the palatoquadrate (pq), ceratohyal (ch), and ethmoid plate (ep) (Figure 1A). Pectoral fin cartilage (pc) did not markedly change (Figure 1A). Calcein staining showed that homozygous *Sox9a* mutation resulted in deformed craniofacial bones, curved spine, and smaller, curved tail bud bone (Figure 1B).

The *Col2a1* gene encodes type II collagen alpha 1 chain, which functions as the principal component of the extracellular matrix in cartilage tissues and is downstream target of Sox9 in mice (Bell et al., 1997). Thus, we explored the effect of homozygous *Sox9a* mutation on *Col2a1* expression in cartilage tissues of Nile tilapia. Whole-mount *in situ* hybridization in larvae at 3 dah showed that *Col2a1a* was highly expressed in the mandible, skull, and pectoral fins in WT Nile tilapia (Supplementary Figure S4), similar to *Sox9a* expression. However, *Col2a1a* expression was undetectable in the heads of the homozygous *Sox9a* mutants (Supplementary Figure S4). Taken together, these findings suggest that Sox9a retains its ancestral role of positively regulating *Col2a1a* expression and cartilage development in Nile tilapia.

We also investigated the effects of *Sox9a* mutation on gonadal development in Nile tilapia. While *Sox9a* expression was present in both the ovary and testis before 30 dah (Supplementary Figure S2), the ovarian factor Cyp19a1a and testicular factor Gsdf were respectively detected in female and male gonads of homozygous *Sox9a* mutants at 5 dah, a critical time for sex determination (Supplementary Figure S5). These results suggest that Sox9a is unlikely to be involved in primary sex determination or gonadal differentiation in Nile tilapia. In addition, as the number of germ cells in both sexes of Nile tilapia does not undergo significant change from 5 to 8 dah, but germ cells in female and male gonads start to proliferate after 8 and 14 dah, respectively (Kobayashi et al., 2008), we further analyzed the effects of the *Sox9* mutation on Nile tilapia germ cell proliferation. Based on immunostaining assay, using an anti-Vasa antibody as a germ cell marker, the number of germ cells in the ovary and testis of homozygous *Sox9a* mutants exhibited no change at 8 and 12 dah but was significantly reduced at 16 dah compared to the WT fish (Figure 1C). Thus, this finding indicates that *Sox9a* mutation represses germ cell proliferation in Nile tilapia gonads.

To determine why *Sox9a* mutation causes a defect in germ cell proliferation in the gonads, we examined whether DNA replication in gonadal cells of Nile tilapia was affected by *Sox9a* mutation. Results from EdU staining revealed that the number of EdU-positive somatic cells surrounding germ cells decreased markedly in both the ovary and testis of homozygous *Sox9a* mutants at 15 dah compared to the WT fish (Supplementary Figure S6). These results indicate that *Sox9a* mutation impairs DNA replication in somatic cells and represses somatic cell proliferation in the gonads of both sexes. However, DNA replication in germ cells was not affected by the *Sox9a* mutation (Supplementary Figure S6). These findings suggest that Sox9a expression in the gonadal somatic cells of both sexes of Nile tilapia promotes somatic cell proliferation, which modulates germ cell proliferation during the juvenile stage.

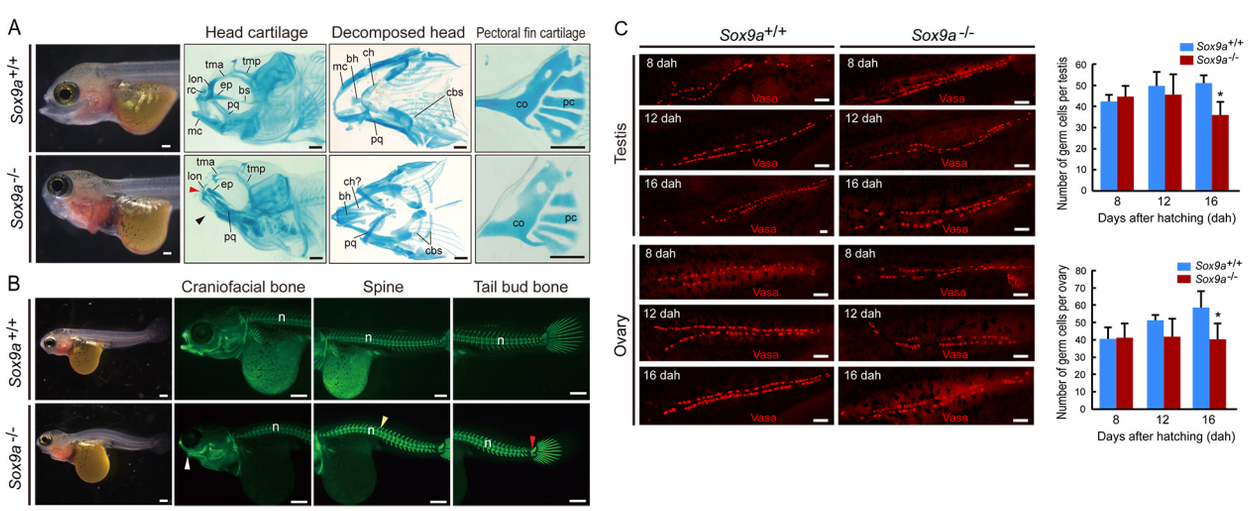


**Figure 1 Effects of *Sox9a* mutation on cartilage development and germ cell proliferation in Nile tilapia**

A: Alcian blue staining analysis of cartilage morphology in *Sox9a*$^{+/+}$ and *Sox9a*$^{-/-}$ Nile tilapia at 10 days after hatching (dah). Black arrowhead, jaw cartilage; red arrowhead, labial cartilage. Full names for all abbreviations are shown in the Alcian blue staining section in the Supplementary Materials and Methods. Scale bar: 50 μm. B: Calcein staining analysis of cartilage development in *Sox9a*$^{+/+}$ and *Sox9a*$^{-/-}$ Nile tilapia at 5 dah. n, notochord. Arrowheads with different colors indicate defects. Scale bar: 100 μm. C: Change in number of germ cells in testis and ovary of *Sox9a*$^{+/+}$ and *Sox9a*$^{-/-}$ Nile tilapia. Scale bar: 50 μm. Data are mean ± standard deviation (*SD*) ($n$=5). For significance test, $^{*}$: $P$<0.05.

In conclusion, our study revealed that Sox9a plays a key role in cartilage development and germ cell proliferation in Nile tilapia, indicating functional conservation and divergence between Sox9a in teleosts and Sox9 in mammals. First, we found that *Sox9a* mutation in Nile tilapia disrupts cartilage development and bone formation, similar to the effects of *Sox9* mutation in mice and zebrafish (Wagner et al., 1994; Yan et al., 2005). Second, while previous studies have shown that Sox9 regulates male sex determination and testis development in mice (Wagner et al., 1994), our study showed that *Sox9a* mutation in Nile tilapia impairs DNA replication in somatic cells and reduces germ cell proliferation in the gonads of both sexes. Based on our findings, along with previous research demonstrating that medaka *Sox9b*, which has a closer phylogenetic relationship with Nile tilapia *Sox9a* than *Sox9b* (Wei et al., 2016), also participates in germ cell maintenance in both sexes (Nakamura et al., 2012), *Sox9a* appears to exhibit a conserved role in teleost germ cell maintenance. However, as all Nile tilapia with the homozygous *Sox9a* mutation died at 20 dah, likely due to developmental defects in craniofacial bones and mandibles, we could not investigate the effects of *Sox9a* mutation on gonadal development, gametogenesis, and fertility after 20 dah. Third, the link between *Sox9a* mutation-induced DNA replication impairment in somatic cells and the reduction in germ cell proliferation remains to be elucidated. Increasing evidence has shown that both somatic granulosa cells in the ovary and somatic Sertoli cells in the testis can produce and secrete factors to regulate germ cell development (Schulz et al., 2010; Vanderhyden, 2002). Therefore, we speculate that Sox9a regulates the proliferation of somatic cells, which may orchestrate the production of somatic cell-derived secretory factors to modulate germ cell proliferation.

To date, loss-of-function assays investigating the pair of *Sox9* duplicates have only been conducted in zebrafish (Yan et al., 2005). Spatiotemporal expression of *Sox9a* and *Sox9b* among teleost fish is complex, showing overlap in some regions as well as region and lineage specificities (Cresko et al., 2003; Wei et al., 2016; Yan et al., 2005). In Nile tilapia, although Sox9a and Sox9b share a high similarity in HMG box domain amino acid sequences, their mRNA expression differs considerably in the ovary and testis (Wei et al., 2016), indicating potential functional partitioning between the *Sox9* duplicates. Further research on the effects of *Sox9b* mutation on different developmental processes in Nile tilapia should enhance our understanding of subfunction partitioning between the *Sox9* duplicates.

## SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

## COMPETING INTERESTS

The authors declare that they have no competing interests.

## AUTHORS' CONTRIBUTIONS

L.W. and D.S.W. designed the project. X.Y.L., Y.H.T., W.Y.D., Y.Z., L.S.W., X.H., Q.P.X., Y.Q.L., and L.D. performed the experiments. L.W., X.Y.L., Y.H.T., and W.Y.D. analyzed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

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