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Molecular cloning and expression analysis of *Sox3* during gonad and embryonic development in *Misgurnus anguillicaudatus*

XIAOHUA XIA, WEIRAN HUO, RUYAN WAN, LINXIA ZHANG, XIAOPEI XIA and ZHONGJIE CHANG*

College of Life Science, Henan Normal University, Xinxiang, Henan, People's Republic of China

ABSTRACT ***Sox3*** **is a single-exon gene located on the X chromosome in most vertebrates. It belongs to the** ***SoxB1*** **subfamily, which is part of the larger** ***Sox*** **family. Previous studies have revealed that** ***Sox3*** **is expressed in many fish species. However, how** ***Sox3*** **influences the development of** ***Misgurnus anguillicaudatus*** **remains unknown. In this study, a** ***Sox3*** **homologue, termed** ***MaSox3,*** **was cloned from the brain of** ***M. anguillicaudatus*** **using homology-based cloning and the rapid amplification of cDNA ends method. Sequence analysis reveals that** ***MaSox3*** **encodes a hydrophilic protein, which contains a characteristic HMG-box DNA-binding domain of 79 amino acids, and shares high homology with** ***Sox3*** **in other species. Additionally, quantitative real-time reverse transcription PCR and** ***in situ*** **hybridization showed that** ***MaSox3*** **is consistently expressed during embryogenesis, with peak expression during the neurula stage and broad expression in the central nervous system. Moreover, tissue distribution analyses have revealed that** ***MaSox3*** **is abundant in the adult brain, the particle cell layer, and the gonad. Additionally, its expression is observed in primary spermatocyte cells, primary oocytes and previtellogenic oocyte cells. Taken together, all of these results suggest that the expression of the** ***MaSox3*** **gene is highly conserved during vertebrate evolution and involved in a wide range of developmental processes including embryogenesis, neurogenesis and gonad development.**

KEY WORDS: *Misgurnus anguillicaudatus, Sox3, embryogenesis, neurogenesis, gonad development*

The *Sox* gene family encodes a large suite of proteins that share over 50% amino acid sequence identity in their HMG-type DNA-binding domain, a domain associated with the mammalian sex determining gene, sex determining region, Y chromosome (*Sry*; Sinclair *et al.,* 1990). The proteins belonging to this family are primarily characterized as chromatin associated protein or transcription factors that use their HMG domain to bind the minor groove of DNA in order to aid in the recruitment and binding affinity of various co-factors to specific DNA regions (Watanabe *et al.,* 2016). In recent years, a series of Sry-related HMG box (*Sox*) genes have been identified in vertebrates, insects and nematodes, and divided into 10 groups, based on their amino acid sequences (*Sox A–J.*; Bowles *et al.,* 2000).

Sox3 is a single-exon gene, belongs to the *SoxB1* (*Sox1*, *Sox2*, *Sox*3 and *Sox19*) subfamily. It is located on the X chromosome of most vertebrates and contains a highly conserved N-terminal HMG-domain and C-terminal domain. Previous work has demonstrated that SOX3 acts as a transcriptional activator for two other *SoxB1* family members, *Sox2* and *Sox3* (Kamachi *et al.,* 1998). In addition, *Sox3* is widely expressed in the early central nervous system of vertebrates and displays some functional redundancy during development with other *Sox* genes (Cheah and Thomas, 2015). *Sox3* is considered an ancestral precursor of the gene *Sry*, which is required for sex differentiation and gonadal development (Weiss *et al.,* 2003). In mice, *Sox3* expression may affect similar developmental pathways as *Sry*; *Sox3* gain-of-function triggers male sex reversal in the uncommitted XX gonad, whereas loss-of-function blocks early spermatogenesis in the postnatal testes germ cells (Laronda and Jameson, 2011).

Abbreviations used in this paper: PCR, polymerase chain reaction.

***Address correspondence to:** Zhongjie Chang. Molecular and Genetic Laboratory, College of Life Science, Henan Normal University, 46# East of Construction Road, Xinxiang, Henan 453007, P. R. China. Tel: +86-373-3326553. E-mail: 13837331530@163.com http://orcid.org/0000-0001-5357-0048

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Fig. 1. Alignment of *Sox3* protein sequences. *The identical, highly conserved and less conserved amino acid residues were indicated by asterisk, colon and dot, respectively. The HMG (high mobility group) box domain is shaded in yellow and the SOXp (SOX transcription factor) domain is boxed. The specificity amino acids were also indicated by arrowheads and underlines. The GenBank accession numbers for the* Sox3 *sequences used for alignment are as follows:* M. anguillicaudatus *(*MaSox3*), Clarias batrachus (*CbSox3*): AIZ03370.1,* Danio rerio *(*DrSox3*): BAD11369.2,* Carassius auratus *(*CaSox3*): ABM55677.1,* Acanthopagrus schlegelii *(*AsSox3*): ABQ96860.1 and* Takifugu rubripes *(*TrSox3*): AAQ18496.1.*

Fishes are the most diverse and species-rich group of vertebrates, serving as an evolutionary link between invertebrate and vertebrates. For instance, research relating to teleost fish has provided several clues to the molecular evolution process of vertebrates. In specific gain- and loss-of-function experiments using the teleost, zebrafish have help to elucidate the role of *Sox3* in both neural fate determination and differentiation (Dee *et al.,* 2008). In *Clarias batrachus*, dynamic expression pattern of *Sox3* in the gonad confirms its potential role in development and germ cell differentiation (Rajakumar and Senthilkumaran, 2014). To further investigate the evolution and functions of *Sox3* in fish development, we decided to analyze the role of *Sox3* in critical developmental processes, including sex determination and differentiation processes. *Misgurnus anguillicaudatus* (Cypriniformes; Cobitidae), a widely distributed teleost native to the eastern coasts of the Asian continent. Specifically, we have quantified expression profiles of *Sox3* during early embryo development and in diverse adult tissues, and analyzed of its cellular distribution in the brain, ovary and testis. These results are necessary to provide fundamental information on both the functional and evolutionary role of *Sox3* across different species.

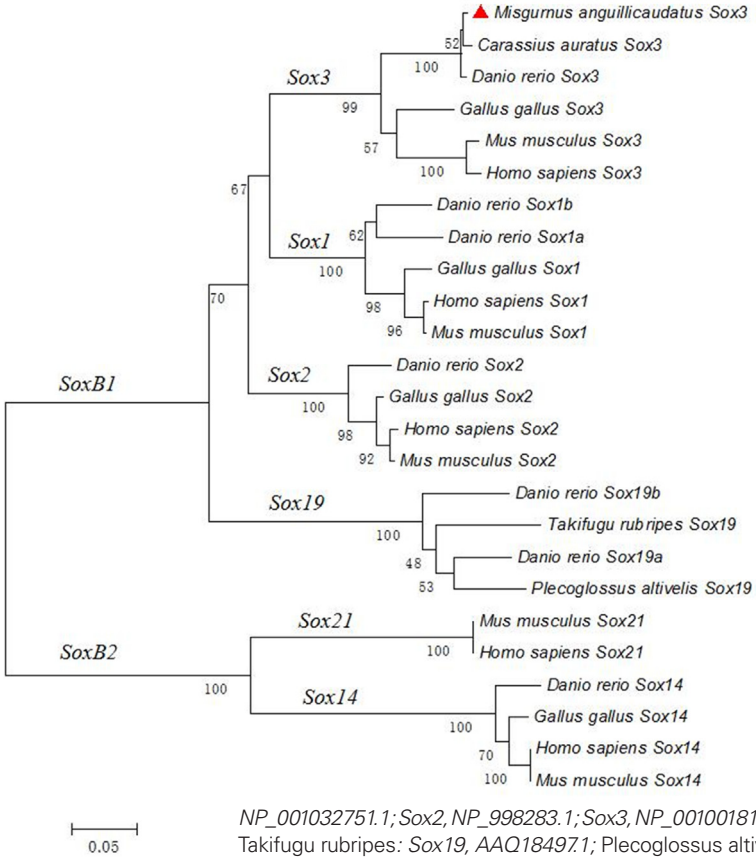

Fig. 2. Phylogenetic tree of MaSox3 in comparison with SoxB proteins in other representative vertebrates using predicted amino acid sequences. *Sox14 and Sox21, belonging to the SoxB2 subgroup were used as outgroup. The numbers in the branches represent the boot-strap value from 1000 replicates obtained using the neighbor-joining method. The scale bar is 0.05. The GenBank accession numbers are as follows:* Homo sapiens*: Sox1, NP_005977.2; Sox2, NP_003097.1; Sox3, NP_005625.2; Sox14, NP_004180.1; Sox21, NP_009015.1;* Mus musculus*: Sox1, NP_033259.2; Sox2, NP_035573.3; Sox3, NP_033263.2; Sox14, NP_035570.1; Sox21, NP_808421.1;* Gallus gallus*: Sox1, NP_989664.1; Sox2, NP_990519.2; Sox3, NP_989526.1; Sox14, NP_990092.1;* Danio rerio*: Sox1a, NP_001002483.1; Sox1b, NP_001032751.1; Sox2, NP_998283.1; Sox3, NP_001001811.2; Sox19a, NP_570983.2; Sox19b, NP_571777.1; Sox14, NP_001032769.1;* Takifugu rubripes*: Sox19, AAQ18497.1;* Plecoglossus altivelis*: Sox19, AHK05948.1;* Carassius auratus*: Sox3, ABM55677.1.*

Results

Cloning and sequence analysis of MaSox3

To clone *MaSox3* from the brain tissue of *M. anguillicaudatus*, we used degenerate primers and the rapid amplification of cDNA ends (RACE) strategy. The *MaSox3* gene (GenBank:

KY704873) is 1863 bp in length and contains a putative open reading frame that encodes a 297 amino acids (AA) protein (Supplementary Fig. 1). The predicted MASOX3 protein is approximately 33.1 kD with a theoretical isoelectric point of 9.63. Furthermore, the protein structure is composed of 40.40% (120 AA) alpha helices 8.75% (26 AA) extended strands, 8.08% (24 AA) beta turns, and 42.76% (127 AA) random coils (Supplementary Fig. 2). The structure analysis also revealed that the MASOX3 protein has three alpha helix structure and two random coil structures (Supplementary Fig. 3).

Homology and phylogenetic analysis

Similar with other species, MASOX3 has the conserved HMG-type DNA-binding domain (Fig. 1), as well as other conserved putative functional domains, such as CYCLIN domain. The Neighbor-joining tree analysis shows MaSox3 was closely clustered with the *Carassius auratus* Sox3 homologues, then with the *Danio rerio* Sox3 homologue (Fig. 2), which is consistent with the classification and evolutionary status for these species.

Hydropathy analysis

The hydropathy profile of the MASOX3 protein was determined by ProtScale program which demonstrated that the arginine$_{103}$ (R) of the putative MASOX3 protein exhibits the highest degree of hydrophilicity (hydrophobic parameter: –3.332), whereas isoleucine$_{222}$ (I) exhibits the highest degree of hydrophobicity (hydrophobic parameter: 1.600) (Fig. 3A). In totality, the SOX3 protein contained more hydrophilic character than hydrophobic areas, similar to the SOX3 protein from *C. auratus* (Fig. 3B).

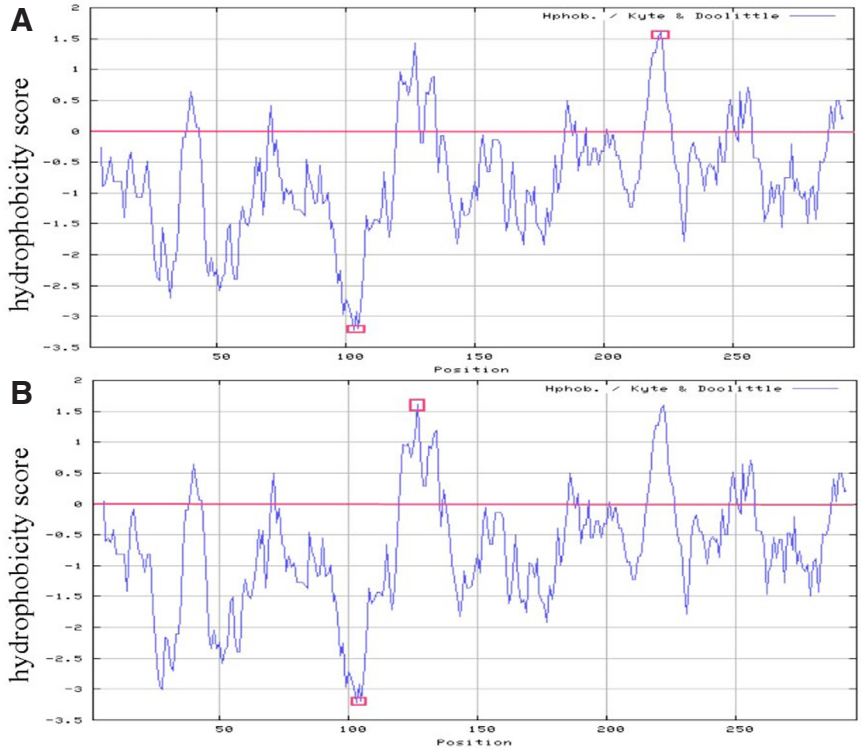

Fig. 3. The hydropathy profile of MaSox3 (A) and *Carassius auratus* Sox3 protein (B). *The hydropathy profile was constructed using the ProtScale program.*

Expression pattern of MaSox3 in different development stages and tissues

To analyze *MaSox3* expression levels in different developmental tissue, we used quantitative real-time reverse transcription PCR (qRT-PCR). We found that *MaSox3* transcripts were highly expressed in gonads ($p < 0.01$); moderately in brain, liver, and heart; and less in the kidney (Fig. 4A). Then we analyzed transcripts during different stages of development. We found that *MaSox3* transcripts initially exhibit low detection levels during gastrula stage of embryo, but then rapidly increase, reaching maximum levels at neurula stage ($p < 0.01$). After reaching its maximum, *MaSox3* transcripts gradually decrease, maintaining stable expression levels until the yolk-sac absorption stage we examined (Fig. 4B).

In situ Hybridization

To examine the distribution of *MaSox3* transcript distribution during embryogenesis, we carried out whole-mount *in situ* hybridization (WISH) using high stringency conditions. *MaSox3* mRNA first accumulated in the eight-cell stage (Fig. 5A), and then accumulated in the hemisphere after the entry into blastula stage (Fig. 5C). After entry into neurula stage, *MaSox3* was expressed broadly in the central nervous system (Fig. 5 E,F). At 20–26 hpf, *MaSox3* was still broadly expressed in the central nervous system including the optic vesicle, which becomes the future retina. *MaSox3* expression level was low in the forebrain–midbrain

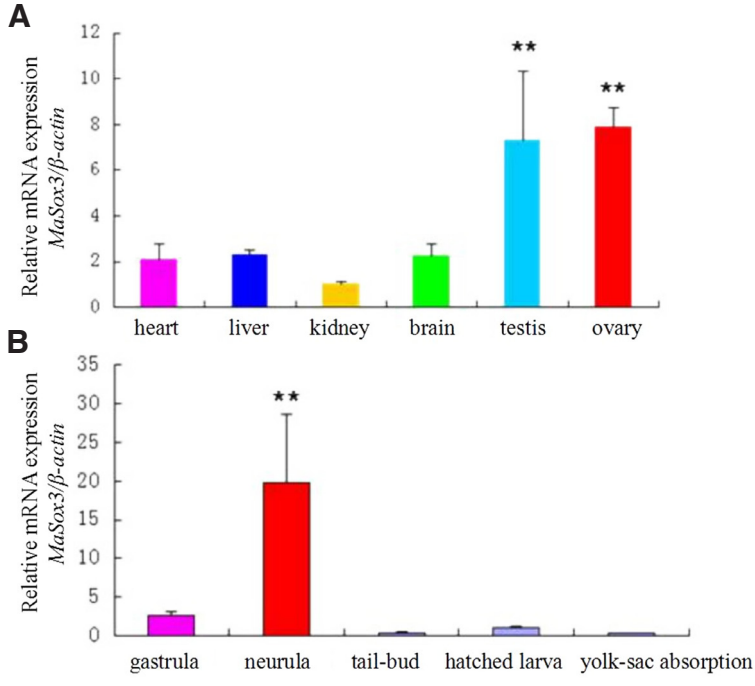

Fig. 4. Expression analysis by quantitative real-time RT-PCR of *MaSox3* in adult tissues (A) and developing embryo (B). *All data were expressed as mean ± SEM (n = 9). Asterisks (*) indicate means with significantly higher* Sox3 *mRNA levels (** $p < 0.01$; * $p < 0.05$).*

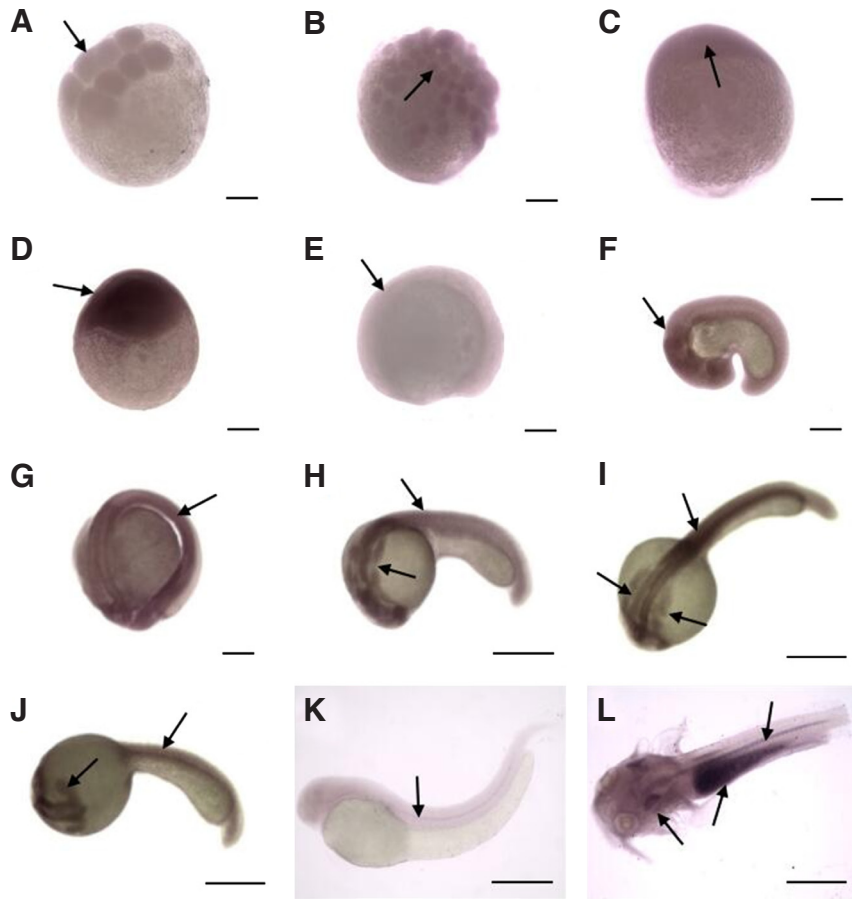

Fig. 5. Whole mount *in situ* hybridization analysis of *MaSox3* during embryogenesis. **(A)** *Eight-cell stage (1 h post fertilization, hpf);* **(B)** *multicellular stage (2 hpf);* **(C)** *blastula stage (3 hpf);* **(D)** *gastrula stage (5 hpf);* **(E)** *neurula stage (11 hpf);* **(F)** *tail-bud formed stage (17 hpf);* **(G)** *otic vesicle formation stage (20 hpf);* **(H)** *otic vesicle formation lateral view (20 hpf);* **(I)** *otic vesicle formation dorsal view (20 hpf);* **(J)** *otic vesicle formation ventral view (20 hpf);* **(K)** *hatched larva (26 hpf);* **(L)** *hatched larva stage dorsal view (26 hpf). Arrow head show obvious hybrid signals. Scale bars (A–G), 200 μm; (H-L), 300 μm.*

and midbrain–hindbrain boundaries, but strong expression was observed in the otic vesicle and viscera (Fig. 5 G–L).

To elucidate localization of *MaSox3* mRNA, we performed *in situ* hybridization (ISH) on testis, ovary and brain sections. In the testis, primordial germ cells develop first into spermatogonia, then into spermatocyte and finally into spermatid. We found accumulation of our probe in both the spermatocytes and spermatids (Fig. 6A). In the ovary, *MaSox3* RNA signal was strongly observed in primary oocyte and previtellogenic oocyte. As the tissue developed, the probe signal decreased, and no signal was detected once the yolk in the previtellogenic oocyte cytoplasm was full (Fig. 6C). In the brain, we observed a strong signal in the particle cell layer, but no obvious signal was observed in the molecular cell layer (Fig. 6E). All results were compared to a *MaSox3* sense RNA probe, which did not display a signal in the ovary, testis and brain (Fig. 6 B,D,F).

Discussion

Previous studies have revealed that *Sox3* is widely expressed in the early central nervous system of vertebrates, and performed very important functions during embryo development, embryonic and adult neurogenesis, sex differentiation, and gonadal development (Rogers *et al.,* 2013; Cheah and Thomas, 2015). Here, we have isolated and characterized the full-length cDNA sequence of a *Sox3* homolog, *MaSox3* in *M. anguillicaudatus*. Similar to other reported *Sox* genes, *MaSox3* contains the highly conserved HMG-box DNA binding domain, suggesting it is structurally conserved during vertebrate evolution (Focareta and Cole, 2016).

Additionally, qRT-PCR result shows that *MaSox3* is widely expressed during *M. anguillicaudatus* embryonic development, from gastrula stage to yolk-sac absorption stage, consistent with Japanese flounder and zebrafish, but different from the red-spotted grouper (Yao *et al.,* 2003; Gao *et al.,* 2015). WISH results showed that *MaSox3* is consistently expressed during embryogenesis. Especially, *MaSox3* is expressed throughout the pluripotent ectodermal cells of blastula embryos, which is associated with germ layer differentiation, histogenesis and organogenesis occur during embryonic development. These results suggested that *Sox3* is involved in early embryonic development process (Rogers *et al.,* 2013).

Previous studies have revealed that *Sox3* regulation is region-specific in the developing nervous system, and plays different roles in the dorsal telencephalon and hypothalamus (Rogers *et al.,* 2013). In zebrafish, knockdown of *Sox3* reduces the size of the central nervous system and subsequently inhibits some aspects of neurogenesis (Dee *et al.,* 2008). Our study suggests that the maximum level of *MaSox3* occurs during the neurula stage of embryogenesis. Additionally, *MaSox3* is highly expressed in brain tissue, similar to what is seen in Japanese flounder (Gao *et al.,* 2015). Moreover, *in situ* hybridization results revealed that the highest expression of *MaSox3* appeared in brain particle cell layer, and broadly expressed in central nervous system including the midbrain and hindbrain. All of these results suggest that *Sox3* is critical for neural development in vertebrates (McAninch and Thomas, 2014).

Sox3 is reported to be an analog of *SRY* in testis and associated with mammalian testis development and differentiation (Rajakumar and Senthilkumaran, 2014). Also, *Sox3* has been found to be associated with male sex differentiation in *Oryzias dancena* (Takehana *et al.,* 2014). The present study shows *MaSox3* is highly expressed in the ovary and testis, and does not exhibit a dimorphic expression pattern. In species-rich fish, diverse expression profiles reveal a distinct *Sox* protein role in either testicular or ovarian development, such as red-spotted grouper, black porgy and catfish (Rajakumar and Senthilkumaran, 2014; Gao *et al.,* 2015). The gonad section using *in situ* hybridization revealed that a positive probe signal was observed in primary spermatocyte cell, primary oocyte and previtellogenic oocyte. In mouse, *Sox3* has been demonstrated to be important for oocyte development, testis differentiation as well as gametogenesis (Weiss *et al.,* 2003). Similarly, *MaSox3* was not expressed during gonadal determination but becomes expressed

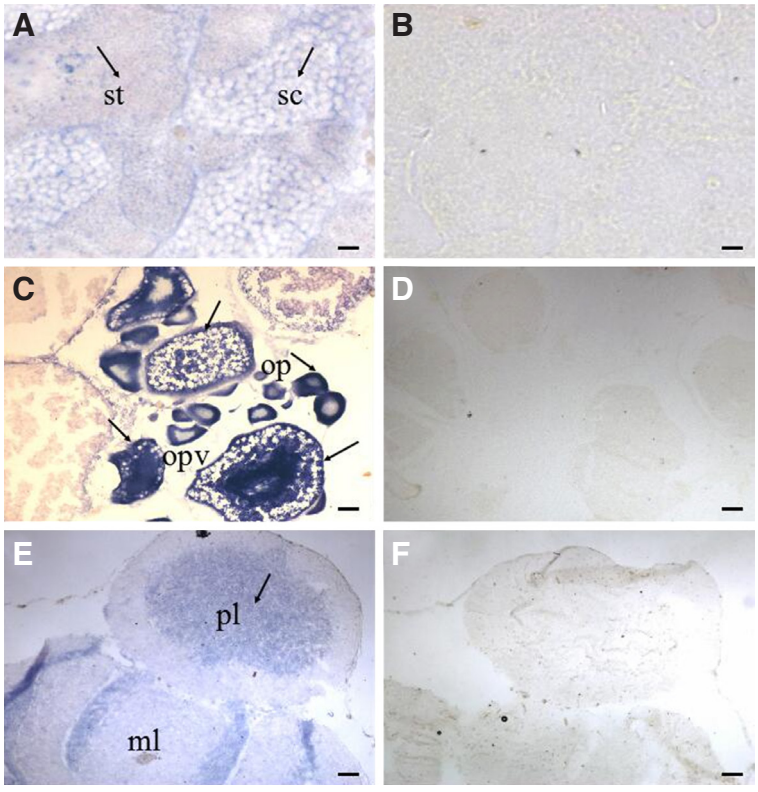

Fig. 6. Expression analysis of *Sox3* by *in situ* hybridization to tissue sections. *Testis* **(A,B)**, *ovary* **(C,D)**, *brain* **(E,F)**. *Anti-sense* Sox3 **(A,C,E)** *and Sense probing as control* **(B,D,F)**. *sc, spermatocyte; st, spermatid; op, primary oocyte stage; opv, previtellogenic oocyte stage; pl, granulosa cell layer; ml, molecular cell layer. Positive signals are shown as black arrows. Scale bars, 50 μm.*

during oocyte development and male testis differentiation and gametogenesis.

Gonad development and maturity is affected by many factors such as gene expression and environmental cues, which involve a complex network of signal molecules. For instance in fish, gonad development is regulated primarily by the hypothalamic–pituitary–gonadal (HPG) axis of the neuroendocrine system (Chen *et al.,* 2013). Our study suggests that *MaSox3* gene may play an important roles during early embryonic development, the formation and development of both the nervous system and gonad development, especially in complex regulatory mechanisms associated with the HPG axis. These results therefore provide fundamental information regarding the function of *Sox3* in teleost fish. Further studies must be carried out to continue elucidating the precise role and mechanism of *Sox3* in fish, especially in regards to complex regulatory mechanisms associated with the HPG axis.

Materials and Methods

Samples

Adult *M. anguillicaudatus* were collected from the wetlands in the old course of the Yellow River, Yanjin County (Henan, China). Animal maintenance and handling procedures followed the recommendations of the Association of Animal Behaviour and national regulations (Elsevier, 2012). Fish spawning and spermiation were artificially induced using intramuscular injection of human chorionic gonadotropin hormone. Three groups consisting of 15–20 zygotic embryos were sampled at the following developmental stages: gastrula, neurula, tail-bud formed, hatched larva and yolk-sac absorption stages were sampled. Adult individuals were acclimatized in a laboratory environment for 48 h before treatment, and then six individuals were randomly selected for the sampling the following tissues: heart, liver, kidney, brain and gonads (ovary and testis). Tissue samples were immediately frozen in liquid nitrogen, and then embryo and larval samples were immersed in 1 mL of RNAwait liquid (Solarbio, Beijing, China) and stored at –80°C until RNA isolation.

RNA extraction and cDNA synthesis

For total RNA extraction, mature adult tissues or embryos/larvae were homogenized and subjected to TRIzol reagent (RNA Extraction Kit, Invitrogen, CA, USA) according to the manufacturer's manual. First-strand cDNA preparation was carried out with Prime Script reverse transcriptase (Takara, Dalian, Japan).

Molecular cloning of Sox3 gene

Sox3 sequences were retrieved from GenBank (www.ncbi.nlm.nih.gov) and multiply aligned by DNAMAN. Degenerate primers were designed based on the conserved regions of the HMG domain (Table 1), and then the resulting PCR product was sequenced. Based on the HMG-box sequence of the *MaSox3* gene, gene-specific primers were designed for the RACE (Table 1). 3' RACE cDNA and 5' RACE cDNA were synthesized using the RACE Core Set (TaKaRa). PCR product was cloned into the pGEM-T Easy Vector (TaKaRa), detected, and sequenced.

Sequence analysis

The full-length cDNA sequence of *MaSox3* was assembled using overlapping regions of each fragment. Both the nucleotide sequence and deduced amino acid sequence were compared with their homologous in other species using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignment of *MaSox3* was performed with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/). The potential protein domain was predicted via SMART server (http://smart.embl-heidelberg.de/). A phylogenetic tree was constructed by Molecular Evolution Genetics Analyses version 6.0 using neighbor-joining method. The evolutionary distance between *SoxB* sequences was calculated using p-distance and gaps were removed by pair-wise detection, using default parameters. To evaluate the tree topological stability, 1000 bootstraps replicates were made. The Physico-chemical parameters of the deduced MASOX3 protein were analyzed by ProtParam tool at ExPASy (http://expasy.org/tools/protparam.html). The secondary structures were predicted with SOPMA software (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_ sopma.html). The three-dimensional structure of deduced MASOX3 protein was predicted by Swiss-Model (https://swissmodel.expasy.org/). The ProtScale program (http://expasy.org/tools/protscale.html) was used to analyze the protein hydropathy profile.

TABLE 1

PRIMERS USED IN GENE CLONING AND RT-PCR

Usage	Primer name	Primer sequence (5'→3')
DegeneratePCR	HMG-F HMG-R	ATGTA (T/C) A(A/G)CATGATGGA(A/G)ACCG CTTTCTT(GC)AGCA(GA)(CG)GTCTTGGTC
3'RACE	3'*Sox3* outer 3'*Sox3* inner	CCAAGCGGTTACGAGCCAT GAAGACCAAGACCGTGCTCAAG
5'RACE	5'*Sox3* outer 5'*Sox3* inner	GGCACCGAGACGCTTGCT CTGATTTCGGAGTTGTGC
Quantitative real-time RT-PCR	*Sox3*-F *Sox3*-R *β-actin*-F *β-actin*-R	CGTGCTCAAGAAAGACAAG ATGCTGGGATGCTGAGGGTAG AGAGAGAAATTGTCCGTGAC GCCAATGGTGATGACCTGT
ISH	ISH-*Sox3*-F ISH-*Sox3*-R	CGTGCTCAAGAAAGACAAG CTGCGTGTATGCTGGTGAC

MaSox3 expression analysis with qRT-PCR

To further quantify the expressions of *MaSox3* in various tissues and different development stages, qRT-PCR were performed using the specific primers shown in Table 1. Reverse transcription products of each sample were properly diluted as templates, and three biological replicates were tested and each sample was assayed in triplicate to ensure reproducibility. β-*actin* was used as control to normalize data from different samples (Xia *et al.,* 2017). The $2^{-\Delta\Delta Ct}$ method was used to analyze the expression levels of *MaSox3* (Livak *et al.,* 2001). All data were expressed as the mean ± standard error of the mean (SEM). The program SPSS V.16 was used for the one-way analysis of variance. And p-values of < 0.01 were considered as statistically significant.

In situ hybridization

The fragment amplified by 3′ RACE was cloned into the pGEM-T vector (Promega) and linearized as template for *in vitro* transcription to generate antisense or sense digoxigenin-UTP labeled RNA probes (DIG RNA labeling kit; Roche, shanghai). WISH and ISH were performed as previously described with minor modifications (Xia *et al.,* 2013; Gao *et al.,* 2015). The sections were observed and photographed with a Nikon Eclipse Ti-U microscope (Nikon, Tokyo, Japan).

Acknowledgments

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