Isolation of *sox9* duplicates in catfish: localization, differential expression pattern during gonadal development and recrudescence, and hCG-induced up-regulation of *sox9* in testicular slices

Kavarthapu Raghuveer and Balasubramanian Senthilkumaran

Department of Animal Sciences, School of Life Sciences-Centre for Advanced Studies, University of Hyderabad, PO Central University, Hyderabad 500 046, Andhra Pradesh, India

Correspondence should be addressed to B Senthilkumaran; Email: bsksl@uohyd.ernet.in

Abstract

In vertebrates, *sox9* is a transcription factor that plays a crucial role in testicular development and chondrogenesis. Here, we report cloning of isoforms of *sox9* (*sox9a* and *sox9b*) from air-breathing catfish *Clarias gariepinus*, which undergoes an annual reproductive cycle. Tissue distribution pattern showed differential expression of *sox9* duplicates, wherein both forms were highly expressed in brain and gonads. Furthermore, we observed a dimorphic expression pattern of *sox9a* and *sox9b* in both adult and developing gonads using RT-PCR, indicating that *sox9a* retained its function in testis while *sox9b* might have a new role to play in ovary. Changes in *sox9* mRNA levels using real-time quantitative PCR (qRT-PCR) during the seasonal reproductive cycle revealed that *sox9a* transcript in testis was abundant during testicular recrudescence (during spermatogenesis), and its expression significantly decreased during spawning and post-spawning phases. Furthermore, treatments of human chorionic gonadotropin and 11-ketotestosterone *in vitro* up-regulated *sox9a* mRNA levels in the testicular slices at 12 and 24 h time points, suggesting that gonadotropins might stimulate *sox9* expression. These results suggest that *sox9* might have a plausible role in the entrainment of the testicular cycle. In contrast, during the ovarian cycle, *sox9b* mRNA levels gradually declined from preparatory to post-spawning phases. Immunohistochemical (IHC) data showed that, in testis, *sox9* is detectable in Sertoli and spermatogonial cell types except spermatid/spermatozoa. In the ovary, it is localized in the ooplasm of primary and pre-vitellogenic oocytes. These results were further confirmed by whole-mount IHC and qRT-PCR.

Reproduction (2010) 140 477–487

Introduction

The SOX (SRY-related high mobility group (HMG) box) gene family encodes several transcription factors that possess a DNA-binding motif known as the HMG domain (Sinclair *et al.* 1990). The HMG domain of all SOX proteins contains 79 amino acid residues, which is highly conserved across different phyla. SOX9 was first identified as the gene responsible for autosomal XY sex reversal associated with campomelic dysplasia, a human skeletal malformation syndrome (Foster *et al.* 1994, Wagner *et al.* 1994). Sox9 is the direct target of the testicular sex-determining gene, Sry (Sekido & Lovell-Badge 2008). Sox9 transcription is up-regulated in the presence of Sry in developing pre-Sertoli cells during male development (Kent *et al.* 1996, Sekido *et al.* 2004). However, Sox9 overexpression in the genital ridges resulted in normal male development in transgenic mice lacking Sry (Bishop *et al.* 2000, Qin & Bishop 2005). In mammals, birds, and turtles, expression of *sox9* is up-regulated in the developing male genital ridges

during testicular differentiation (Kent *et al.* 1996, Morais da Silva *et al.* 1996, Moreno-Mendoza *et al.* 1999). In mice, ectopic expression of Sox9 in XX gonads resulted in the formation of testis (Vidal *et al.* 2001). These results together suggest for a crucial role of *sox9* in testicular development of several vertebrates. In addition to this, *sox9* is also involved in the regulation of many developmental events such as notochord maintenance (Barrionuevo *et al.* 2006), prostate development (Thomsen *et al.* 2008), and cartilage formation in vertebrate species, including teleosts (Bi *et al.* 1999, Chiang *et al.* 2001, Yan *et al.* 2002).

To date, the *sox9* gene has been identified in several vertebrate species including mammals (Foster *et al.* 1994, Wagner *et al.* 1994, Kent *et al.* 1996), birds (Morais da Silva *et al.* 1996), reptiles (Moreno-Mendoza *et al.* 1999, Western *et al.* 1999, Choudhary *et al.* 2000), amphibians (Takase *et al.* 2000), and fishes (Takamatsu *et al.* 1997, Chiang *et al.* 2001, Yokoi *et al.* 2002). Two different forms of *sox9* genes have been identified in

teleosts like zebrafish, rice field eel, fugu, and medaka (Chiang *et al.* 2001, Zhou *et al.* 2003, Koopman *et al.* 2004, Klüver *et al.* 2005). These two forms of *sox9* gene were found to be orthologs of the tetrapod *Sox9* gene that arose during a whole-genome duplication event (Amores *et al.* 1998, Postlethwait *et al.* 2004). In rainbow trout, *sox9* was expressed in testis (Takamatsu *et al.* 1997), while in medaka, *sox9* was expressed in ovary (Yokoi *et al.* 2002). In zebrafish, *sox9a* was expressed in testis, as in mammals, and *sox9b* in ovary (Chiang *et al.* 2001). In rice field eel, two *sox9a* genes (*sox9a1* and *sox9a2*) were expressed in testis, ovary, and ova-testis (Zhou *et al.* 2003). In medaka, *sox9a2* was isolated, and found to be expressed at the highest level in adult testis (Nakamoto *et al.* 2005). In common carp, *sox9b* was expressed in testis (Du *et al.* 2007). These results suggest that the expression of *sox9* in gonads seems to be somewhat diversified in teleosts. Most of these studies were done during early gonadal development in daily or fortnight breeders like zebrafish, medaka, and tilapia. In this regard, catfish (*Clarias gariepinus*) is an annual breeder, which undergoes seasonal pattern of gonadal attenuation and recrudescence that may provide interesting highlights to understand the role of *sox9* not only during development but also during recrudescence. In the present study, we cloned full-length cDNAs of *sox9a* and *sox9b* from catfish gonads, and analyzed their expression pattern in different stages of gonadal development and recrudescence. Extensive analysis was also made to localize Sox9 protein for the first

time in developing and adult gonads. We also studied the expression pattern of *sox9* in human chorionic gonadotropin (hCG) and 11-ketotestosterone (11-KT) -treated testicular slices at different time intervals by an *in vitro* culture.

Results

Cloning of *sox9a* and *sox9b* from catfish gonads

Two distinct partial cDNA fragments of *sox9* were identified in catfish: *sox9a* (269 bp) from testis and *sox9b* (266 bp) from ovary using RT-PCR with degenerate primers. The 5' and 3' ends of these partial cDNA fragments were amplified using RACE PCR. The full-length catfish *sox9a* cDNA isolated (2065 bp) from testis had an open reading frame (ORF) of 1383 bp encoding a putative protein of 460 amino acid residues. Catfish *sox9b* cDNA (2059 bp) obtained from ovary had an ORF of 1377 bp encoding a putative protein of 458 amino acid residues. ClustalW alignment of these two duplicates, *sox9a* and *sox9b* from catfish, showed 62% homology between them at the amino acid level (Fig. 1). Multiple sequence alignment reveals that the HMG box domain is highly conserved, with more than 96% sequence identity with other vertebrates. Sequence homology among *sox9* from different species other than the HMG domain showed an overall 70–85% sequence identity among all other known vertebrate SOX9 proteins. Phylogenetic analysis using neighbor-joining method revealed that the catfish *sox9a* was closely

Figure 1 ClustalW alignment of deduced amino acid sequences of catfish *sox9a* and *sox9b*. This alignment was created using the ClustalW alignment software version 1.82 from European Bioinformatics Institute website (<http://www.ebi.ac.uk/Tools/clustalw2>). Arrows indicate intron positions. Solid lines indicate conserved HMG box region, and dotted lines indicate transactivation domain.

related to rainbow trout, while catfish *sox9b* was clustered with *sox9b* of carp and zebrafish (Fig. 2). The nucleotide sequence data of *sox9a* and *sox9b* have been submitted to GenBank. The accession number of catfish *sox9a* is HM149258 and *sox9b* is HM149259.

Tissue distribution pattern of *sox9* duplicates by RT-PCR

Tissue distribution analysis by RT-PCR showed that the expression of *sox9a* (Fig. 3A) was confined to few tissues like brain, heart, gill, liver, kidney, and testis. On the other hand, *sox9b* transcript (Fig. 3A) was detectable in brain, heart, liver, ovary, and spleen. However, in both cases, *sox9* was abundantly expressed in brain and gonads. Interestingly, *sox9a* and *sox9b* depicted sexual dimorphism.

Dimorphic expression pattern of *sox9a* and *sox9b* in adult and developing gonads

Further analysis of sexual dimorphism of *sox9* by semi-quantitative RT-PCR endorsed that *sox9a* was detectable in adult testis while *sox9b* was expressed in adult ovary (Fig. 3B). This kind of expression pattern of *sox9* duplicates was observed in developing gonads at different age groups (50, 75, 100, and 150 days post

Figure 2 Phylogenetic tree showing the evolutionary status of catfish *sox9a* and *sox9b*. The tree was constructed using the neighbor-joining method, and a bootstrap analysis with 1000 replicates was used to assess the strength of nodes in the tree. Phylogenetic analysis was done using CLC Main workbench 5 software. GenBank accession numbers are as follows: human (CAA86598), mouse (NP_035578), chicken (AAB09663), alligator (AAD17974), rainbow trout (BAA24365), pejerrey (AAP84605), zebrafish *sox9a* (AF277096), carp *sox9a* (AAZ07990), fugu *sox9a* (AAQ18507), medaka *sox9a* (AAX62152), rice field eel *sox9a1* (AAK59254), zebrafish *sox9b* (AF277097), carp *sox9b* (AAX56088), fugu *sox9b* (AAQ18508), medaka *sox9b* (AAX62151), and rice field eel *sox9a2* (AAK59255).

Figure 3 (A) RT-PCR analysis of *sox9a* and *sox9b* in different tissues of adult catfish. (B) Semi-quantitative RT-PCR analysis ($n=3$) of *sox9* duplicates in adult testis and ovary. (C) Semi-quantitative RT-PCR ($n=3$) amplification of *sox9a* and *sox9b* in developing gonads at different age groups of catfish (50, 75, 100, and 150 days post hatching). β -actin was used as an internal endogenous control.

hatching (dph)) in catfish, wherein *sox9a* expression was seen in developing testis but not in developing ovary, and the reverse is true for *sox9b* (Fig. 3C).

Expression of *sox9* in different phases of testicular and ovarian cycles

Real-time quantitative PCR (qRT-PCR) analysis revealed that *sox9a* expression was significantly higher during preparatory and pre-spawning phases when compared with spawning and post-spawning phases of testicular cycle (Fig. 4A). This indicates that *sox9a* transcripts are abundantly expressed during active spermatogenesis, i.e. in preparatory and pre-spawning phases, wherein spermatogonia and spermatocytes (SC) are more in number (Swapna *et al.* 2006), while *sox9b* transcript was abundantly expressed during preparatory phase, and later its expression was drastically reduced in pre-spawning, spawning, and post-spawning phases of the ovarian cycle (Fig. 4B).

Effect of hCG treatment in vitro on *sox9a* expression in the testicular slices

To test whether *sox9a* expression can be regulated by gonadotropins, hCG treatment was done, as it is functionally similar to LH. Expression of *sox9a* was analyzed in testicular slices (late preparatory phase of mature fish) that were collected at different time intervals after the treatment of hCG *in vitro* using qRT-PCR.

Figure 4 (A) qRT-PCR analysis of *sox9a* expression during different phases of the testicular cycle in catfish. The relative expression of *sox9a* normalized with β -actin in samples was calculated using comparative C_t method. Data for real-time PCR were expressed as mean $\pm$ S.E.M. ($n=5$). Different letters on the bars indicate significant increase in the mRNA levels in preparatory and pre-spawning phases from that of spawning and post-spawning phases ($P<0.05$, ANOVA followed by SNK test). Same letters on the bars indicate that the differences were not significant. (B) qRT-PCR analysis of *sox9b* expression during different phases of the ovarian cycle in catfish. Other details are as in Fig. 4A.

The expression of *sox9a* was significantly increased at 12 h ($*P<0.05$) and peaked at 24 h ($**P<0.001$) after hCG treatment (Fig. 5).

Effect of 11-KT treatment in vitro on *sox9a* expression in the testicular slices

Since hCG is known to induce androgen production in testis, we intend to study the effect of 11-KT (a potent androgen in fish) on *sox9a* expression in testicular slices. The qRT-PCR analysis revealed that the 11-KT treatment has significantly increased the *sox9a* transcripts in testicular slices (late preparatory phase of mature fish) at 12 h ($*P<0.05$) and 24 h ($**P<0.001$) time points when compared with 0 h (Fig. 6).

Figure 5 qRT-PCR analysis showing *sox9a* mRNA levels in the testicular slices treatment with hCG *in vitro* at different time intervals. *sox9a* mRNA levels were significantly increased after hCG induction *in vitro* at 12 and 24 h time points compared with the 0 h time point. $*P<0.05$; $**P<0.001$ (ANOVA followed by SNK test, $n=3$).

Expression pattern of *sox9b* in different stages of follicles

We collected four different stages of follicles (Fig. 7A) from catfish ovary during pre-spawning period: stage I (perinucleolar/primary oocytes), stage II (pre-vitellogenic oocytes), stage III (vitellogenic oocytes), and stage IV (post-vitellogenic oocytes). qRT-PCR was performed to quantify the *sox9b* transcript abundance in these four stages of oocytes. The real-time PCR results (Fig. 7B) showed that the transcript was abundantly expressed in stage I oocytes, moderately low in stage II oocytes ($*P<0.05$), and its expression was drastically reduced in stage III and IV oocytes ($**P<0.001$).

Localization of Sox9 in developing and adult gonads of catfish

Western blot analysis revealed an expected band of ~ 51 kDa in testis and ovarian tissue homogenates of catfish. This band corresponds to the Sox9 protein, which encodes a 51 kDa putative protein (Fig. 8). Absence of immunoreactivity using the antibody pre-absorbed with Sox9 synthetic peptide confirms that the primary antibody is indeed specific to Sox9 protein

Figure 6 qRT-PCR analysis showing *sox9a* mRNA levels in the testicular slices treatment with 11-KT (100 ng/ml) *in vitro* at different time intervals. *sox9a* mRNA levels were significantly increased after hCG induction *in vitro* at 12 and 24 h time points compared with the 0 h time point. $*P<0.05$; $**P<0.001$ (ANOVA followed by SNK test, $n=3$).

Figure 7 (A) Hematoxylin–eosin-stained section of mature catfish ovary showing different stages of oocytes. Scale bar indicates 50 μm. (B) qRT-PCR analysis showing the expression pattern of *sox9b* in different stages of oocytes. The relative expression of *sox9b* in different stages was calculated using comparative C_t method. *sox9b* mRNA levels were significantly lower in stage II, III, and IV oocytes, when compared with stage I oocytes. * $P < 0.05$; ** $P < 0.001$ (ANOVA followed by SNK test, $n = 5$).

in catfish (Fig. 9A). Immunohistochemical (IHC) analysis in the preparatory phase of adult testis revealed that Sox9 immunoreactivity was observed in Sertoli and spermatogonial cell types such as primary spermatogonia (SG1), secondary spermatogonia (SG2), and SC except spermatid/spermatozoa (SP; Fig. 9B–D). In the case of adult ovary, Sox9 immunoreactivity was restricted to ooplasm of stage I and stage II oocytes (Fig. 9E). This was further confirmed by the whole-mount IHC in different follicular stages (oocytes), wherein the signal was abundant in stage I and stage II oocytes (Fig. 9F and G). No signal was detectable in stage III and stage IV oocytes (Fig. 9G). No immunoreactivity was observed with pre-absorbed antibody (negative control, Fig. 9H). During the critical period of gonadal differentiation in catfish, Sox9 protein was detectable in germ and supporting cells of undifferentiated gonad at 40 dph (Fig. 8I). Sox9 immunoreactivity was prominent in the

primary oocytes (stage I) and oogonia of differentiated ovary at 50 dph and in stage I and II oocytes of juvenile ovary at 100 dph (Fig. 9J and K), whereas in developing testis at 100 dph, it was observed in the Sertoli and germ cells present in cysts (Fig. 9L).

Discussion

In the present study, we report cloning of two duplicate orthologs of *sox9*, named *sox9a* and *sox9b*. Both the isoforms contain the Sox gene signature domain HMG, which is highly conserved across different vertebrates. Recent studies in teleosts like zebrafish, medaka, rice field eel, and carp also reported two forms of *sox9* (Chiang *et al.* 2001, Zhou *et al.* 2003, Klüver *et al.* 2005, Du *et al.* 2007). Phylogenetic analysis using neighbor-joining method revealed that the isolated catfish *sox9* duplicates showed high homology to its counterpart in yellow catfish and zebrafish. Previously, Klüver *et al.* (2005) reported in medaka that the two *sox9* genes are co-orthologs of the single tetrapod Sox9. This was reliable with whole-genome duplication in the ray-fin lineage (Amores *et al.* 1998, Postlethwait *et al.* 2004) that might have promoted lineage divergence by partitioning different ancestral gene subfunctions among co-orthologs of tetrapod genes (Lynch & Force 2000, Cresko *et al.* 2003). Zhou *et al.* (2003) also reported that the *sox9* gene was duplicated during the evolution of rice field eel. The duplicated copies of the *sox9* gene showed similar kind of expression pattern in testis, ovary, and ova-testis. Tissue distribution analysis in catfish showed differential expression pattern of *sox9a* and *sox9b*. In catfish, both the isoforms are expressed abundantly in brain and gonads. Semi-quantitative RT-PCR analysis in adult gonads revealed sexual dimorphic expression of *sox9a* and *sox9b* in adult and

Figure 8 Western blot showing Sox9 protein (band) of molecular weight ~51 kDa detectable in testis and ovary of adult catfish, which is consistent with the size, calculated using the LaserGene software (51224.06 Da) from the deduced amino acid sequence of Sox9. Arrow indicates Sox9 protein (band).

Figure 9 Immunocytochemical (IHC) localization of Sox9 protein in adult testis using anti-human Sox9 antibody. (A) Negative control showing Sox9 immunoreactivity was abolished when pre-absorbed antibody was used as the primary antibody. (B) Adult testis section showing Sox9 immunoreactivity in boundaries of lumens filled with spermatids/sperm (SP); arrowheads indicate positive immunoreactivity. (C and D) Adult testis section at higher magnification showing Sox9 signal was detectable in Sertoli and spermatogonial cell types except spermatid/sperm (SP). Arrows indicate the position of the cell types: Sertoli cells (S), primary spermatogonia (SG1), secondary spermatogonia (SG2), spermatocytes (SC), and SP. Counterstained with hematoxylin. (E) IHC detection of Sox9 in different oocytes of mature female. Sox9 protein was detectable only in stage I (primary oocytes) and stage II (pre-vitellogenic oocytes) oocytes of mature ovary using IHC. There was no signal in stage III and stage IV oocytes. (F and G) Whole-mount IHC localization of Sox9 protein in different stages of oocytes. (H) Negative control showing no signal when pre-absorbed Sox9 antibody was used as the primary antibody. (I) Sox9 signal in germ cells (shown by arrow) of undifferentiated gonad at 40 dph. (J) Differentiated ovary at 50 dph. Arrows indicate primary oocytes, arrowheads indicate oogonial cells, and * indicates ovarian cavity. (K) Developing ovary at 100 dph. Arrows indicate primary oocytes and arrowheads indicate pre-vitellogenic oocytes. Inset shows immunoreactivity in stage I and stage II oocytes at higher magnification. (L) Immature testis at 100 dph. Counterstained with hematoxylin. All scale bars indicate 50 μ M.

developing gonads, wherein *sox9a* was preferably expressed in testis, while *sox9b* was abundantly expressed in ovary. This type of differential expression pattern of duplicated genes may attribute to more flexible gene regulation and function during catfish development. A similar kind of expression pattern was also noticed in zebrafish using PCR Southern and *in situ* hybridization (Chiang *et al.* 2001). Sexual dimorphic expression pattern of *sox9a* was observed 25 days after hatching in a monosex population of Nile tilapia (Kobayashi *et al.* 2008). This kind of dimorphic expression pattern of *sox9* duplicates in fish gonads indicates that *sox9a* retained its function as a testis-specific gene, while *sox9b* might have a new role to play in ovarian development. Male-specific expression pattern of *sox9a* was also observed in fresh water and marine turtles suggesting its role in testicular differentiation (Spotila *et al.* 1998, Moreno-Mendoza *et al.*

1999). In contrast, there are a few reports wherein *sox9* duplicates did not show any dimorphic expression in the gonads of medaka and eel (Zhou *et al.* 2003, Klüver *et al.* 2005).

In mammals and birds, *sox9* expression was down-regulated during ovarian differentiation, but its expression was high in developing testis and remains throughout adulthood (Kent *et al.* 1996, Morais da Silva *et al.* 1996). As *sox9* expression still persists after gonadal development, its role during adulthood in teleosts is yet to be defined. The fate of *sox9* in different phases of adult gonads during the reproductive cycle has not been studied in detail so far in any teleosts. To this end, we quantified *sox9* transcript abundance in different stages of testicular and ovarian cycle. Real-time PCR analysis of *sox9a* during testicular cycle showed that the transcript was higher during the period of spermatogenesis, i.e. preparatory and pre-spawning phases, and its expression

decreased gradually thereafter during spermiation and post-spawning phases. This high expression during spermatogenesis is due to a higher number of germ cells expressing *sox9*. In contrast, during the ovarian cycle, expression of *sox9b* transcript was abundant in the preparatory phase when compared with the pre-spawning phase, spawning, and post-spawning phases. Recent report showed that GnRh analog treatment (*in vitro* and *in vivo*) up-regulates *sox3* and *dmrt1* mRNA expression in immature black porgy (Shin *et al.* 2009). In another study, it was observed that FSH could regulate *Dmrt1* expression in postnatal rat testis (Chen & Heckert 2001). In light of this finding, the role of gonadotropins, if any, on the regulation of *sox9* mRNA expression is not known in lower vertebrates. Hence, we studied the expression of *sox9* after hCG induction specifically during testicular recrudescence, as *sox9* expression tends to be at higher levels in preparatory and pre-spawning phases. We observed a significant increase in *sox9a* mRNA in the testicular slices (prepared from testis in late preparatory phase) upon treatment with hCG *in vitro* at 12 and 24 h time points. This result tends to propose a role for gonadotropins in *sox9* regulation, at least in testis. In this regard, LH-like hCG is known to bind to LH receptors, which is expressed by Leydig cells to stimulate androgen production and acts on Sertoli cells to influence spermatogenesis (Skinner 1991). In fish, it is well known that hCG (functionally similar to LH) induces testosterone and 11-KT production *in vitro* (Wade & Van der Kraak 1991). Hence, it is possible that hCG might have induced *sox9a* via androgen production. To test this contention, we also studied the effect of 11-KT on *sox9a* expression in testicular slices (*in vitro* culture). In this study, for the first time, we demonstrated that the treatment with 11-KT significantly elevated the expression of *sox9a* in testicular slices. This may be one of the modes of action for gonadotropins. The androgen receptor present in Sertoli cells is known to regulate specific genes such as *Rhox5/Pem*, which is required for the gametogenesis in mice (Verhoeven *et al.* 2010). However, in this study, the specific mechanism of how androgens regulate *sox9a* expression in Sertoli cells needs to be elucidated. Furthermore, there are reports which clearly show that gonadotropins like FSH can directly act on Sertoli cells and regulate the expression of other genes by activating other transcription factors like cAMP response element-binding protein (CREB) and cAMP response element modulator (CREM; Sassone-Corsi 1995). It has been reported that FSH can regulate the expression of *Dmrt1* in Sertoli cells via the cAMP–PKA pathway by activating CREB or CREM (Chen & Heckert 2001). In humans, it has been shown that the SOX9 promoter is regulated by CREB (Piera-Velazquez *et al.* 2007). These results altogether suggest that gonadotropins can regulate *sox9* expression in Sertoli cells either directly or indirectly.

IHC data in male gonad revealed that Sox9 was localized in Sertoli cells and different stages of germ cells, while spermatid/spermatozoa did not show any signal. A similar localization pattern to that of Sox9 in germ cells was noticed for *Dmrt1* in catfish, zebrafish, red-spotted grouper, and mice (Guo *et al.* 2005, Xia *et al.* 2007, Raghuvveer & Senthilkumaran 2009) and for Sox3 in grouper (Xia *et al.* 2007), which are related to testicular development. This contention requires more validation. Nevertheless, we used a highly specific antibody that detects only Sox9 protein in Sertoli cells of human testis (as per antibody characterization data from the manufacturer). Hence, the observation of Sox9 protein in Sertoli and germ cells in catfish is acceptable technically, and we presume that the detection of Sox9 may be a feature observed in siluriformes like that of *Dmrt1*. However, in zebrafish, *sox9* expression was confined only to Sertoli cells of testis (Chiang *et al.* 2001). The presence of Sox9 protein in oogonia during ovarian differentiation is shown for the first time in any lower vertebrates. This further validates that Sox9 protein is also detectable in germ cells. However, the difference in subcellular localization of male and female germ cells of the protein recognized by the anti-*sox9* antibody is quite striking, as it is found in the nucleus of male germ cells while there is little to no signal in the nucleus of developing oogonia. The apparent reason for low detection of this transcription factor (Sox9 protein) in the nucleus of female germ cell is not clear at the moment. In accordance with our findings, *sox9* transcripts were shown to be detectable mostly in the cytosol of immature oocytes of zebrafish by *in situ* hybridization (Chiang *et al.* 2001). In mature female catfish, *sox9* expression was restricted to perinucleolar stage of developing oocytes and pre-vitellogenic oocytes, but not in mature oocytes. In the whole-mount IHC, Sox9 signal was more in the periphery with less intensity in ooplasm. On the other hand, in the cross section of mature ovary, the immunoreactivity was more clearly evident in ooplasm. The decrease in *sox9* transcript in mature oocytes may be due to the dilution of transcripts with increasing mass/size of mature oocytes that moreover accumulate large amount of vitellogenin and maternal RNAs. These results together indicate that *sox9* may have an important role in early gonadal development and recrudescence.

In conclusion, we identified/isolated *sox9* duplicates (*sox9a* and *sox9b*) in catfish gonads. Dimorphic expression pattern of *sox9* duplicates in developing gonads authenticates an important role for the correlates in gonadal development. Sox9 protein could be localized in Sertoli and germ cells of catfish testis. Its expression was prominent in immature oocytes, while it is negligible in mature oocytes. High expression of *sox9* during gonadal recrudescence indicates its importance in the entraining of the gonadal cycle in teleosts, which may be regulated by gonadotropins.

Materials and Methods

Animals and sampling

Catfish (*Clarias gariepinus*) at different age groups were reared in fresh water tanks under ambient photothermal conditions. Mature spermiating male and gravid female fishes injected with hCG were used for IVF to obtain catfish fries at different age groups. The newly hatched catfish fries were fed with live tubeworms until 3 months. Juvenile fishes of 3–8 months old were fed with live tubeworms besides commercially available fish feed pellets. Adult catfish (1 year old) were reared in the outdoor tanks of the laboratory and fed with minced goat liver *ad libitum* in addition to fish feed. Commonly referred to as the African or air-breathing catfish, this species is abundantly available in ponds and lakes of Hyderabad. The seasonal reproductive cycle of catfish is divided into four phases as follows (Swapna *et al.* 2006): preparatory (February–April), pre-spawning (May–June), spawning (July–October), and post-spawning/regressed phases (November–January). The seasonal reproductive cycle is seen both in wild caught and in natural outdoor tank-maintained fishes. Adult gonads of catfish were collected during reproductive cycle and a part of it was fixed in 4% paraformaldehyde (PFA), while remaining tissue was stored at -80°C for total RNA extraction. As reported earlier, the morphological signs of sex differentiation were completed at 50 dph in catfish (Raghuveer & Senthilkumaran 2009). Juvenile catfish fries at different age groups (50, 75, 100, and 150 dph) were dissected, and gonads were removed using a fine forceps under stereozoom microscope. Gonads (five to ten fishes) of similar age group and same sex were pooled and stored at -80°C for total RNA extraction. Oocytes at different maturation stages were collected from pre-spawning female catfish. Oocytes were separated under stereozoom microscope in F.O. solution (113 mM NaCl, 1 mM KCl, 2 mM CaCl_2 , 5 mM HEPES, pH 7.5) in four different stages: stage I, perinucleolar/primary oocytes ($<50\text{ }\mu\text{m}$); stage II, pre-vitellogenic oocytes (50–100 μm); stage III, vitellogenic oocytes (150–300 μm); and stage IV, post-vitellogenic oocytes (300–800 μm). After separation, total RNA was immediately prepared, without any freezing step.

Cloning of partial cDNA fragments of *sox9a* and *sox9b* from catfish gonads

Total RNA was prepared from adult catfish testis and ovary using the Sigma TRI-reagent method. RT was carried out using superscript-III (Invitrogen) with oligo d(T)₁₈ primers and 5 μg of total RNA at 50°C according to the manufacturer's protocol. PCR amplification was done using a PCR master mix (Fermentas, Glen Burnie, MD, USA) under the following conditions: 94°C (1 min), 55°C (1 min), and 72°C (1 min) for 35 cycles using the *sox9dgFr* and *sox9dgRv* degenerate primers (Table 1) designed based on the available *sox9* nucleotide sequences of other species from NCBI GenBank database. PCR-amplified cDNA fragments were gel purified, cloned in TOPO cloning vector (Invitrogen), and sequenced. The partial cDNA fragments of *sox9a* and *sox9b* obtained from testis and ovary respectively were confirmed using NCBI BLAST search.

Table 1 List of primers used for PCR amplification.

Primers	Nucleotide sequence (5'–3')
<i>sox9dgFr</i>	GC(AC)GA(GA)(CT)T(GC)AGCAAAAC(ATC)C(TG)G
<i>sox9dgRv</i>	CTGGA(TA)GC(TG)GG(CGA)A(AG)TCGG
<i>sox9a5' GSP1</i>	ACAAATGGCCGCTTCTCCCCTTC
<i>sox9b5' GSP1</i>	TGCGTGTGCTCGGGACCCTCTTCC
<i>sox9a5' GSP2</i>	AGCCGCCAGAGTTTGCCCGG
<i>sox9b5' GSP2</i>	TCCTTCGCCGTGGCTGGTACT
<i>sox9a3' GSP1</i>	AAGGGCGGCCAGGGTAAACCG
<i>sox9b3' GSP1</i>	GCATCAGCCGGCCTCAGTACG
<i>sox9a3' GSP2</i>	AGCCCTACAGGGAGCCGACTC
<i>sox9b3' GSP2</i>	GAGTTCTAGCCAGAGGCCCATG
<i>sox9aF</i>	ACCCAGAGGCCCATGTACAC
<i>sox9aR</i>	CTGGTGGAGGGAGTGGAC
<i>sox9bF</i>	CGCAGTTACCGTTGGCTCTG
<i>sox9bR</i>	GCTGCAGGTTGAAGGAGCCA
β -actinRTF	GCAGAGCTCAGCAAAACCCGG
β -actinRTR	GCTGGAAGCGGGAGAGTCGG
<i>sox9aRTF</i>	TCTGGCGGCTGCTGAATGAAGG
<i>sox9aRTR</i>	CTCGGTATCCTCGGTTTCACC
<i>sox9bRTF</i>	GAGACCCAGTCAGGCCACAG
<i>sox9bRTR</i>	AGGGTCTCGATGTGGGCCA

RACE

Full-length cDNAs of *sox9a* and *sox9b* were obtained from testis and ovary respectively using 5' and 3' RACE strategies. Gene-specific primers (GSP) for RACE were designed based on the sequence information of partial cDNA fragments of *sox9a* and *sox9b* (Table 1). SMART RACE cDNA amplification kit (Clontech) was used to prepare 5' and 3' cDNA templates from testis and ovary total RNA according to the manufacturer's protocol. To obtain 5' and 3' ends of *sox9* duplicates, we performed touchdown PCR using *sox9a* and *sox9b* 5' and 3' GSP1 primers (Table 1), and universal primer A mix with the RACE templates under following cycling conditions: 94°C (30 s) and 72°C (2 min) for 5 cycles, 94°C (30 s) and 70°C (2 min) for 5 cycles, 94°C (30 s), and 68°C (30 s) and 72°C (2 min) for 25 cycles. After the primary PCR, nested PCR was performed using *sox9a* and *sox9b* 5' and 3' GSP2 primers (Table 1), and nested universal primer with diluted primary PCR products. The nested PCR conditions were as follows: 94°C (30 s), 65°C (30 s), and 72°C (2 min) for 35 cycles. All the nested PCR-amplified DNA fragments were gel purified, cloned in TOPO cloning vector (Invitrogen), sequenced, and analyzed using LaserGene software 3.05 (DNASTAR, Madison, WI, USA). The nucleotide and deduced amino acid sequences of *sox9a* and *sox9b* cDNAs were verified using the NCBI BLAST search.

Tissue distribution by RT-PCR

Tissue distribution pattern of *sox9a* and *sox9b* in adult catfish was carried out using RT-PCR. For this, total RNA was extracted from different tissues (brain, spleen, gill, heart, intestine, kidney, liver, testis, and ovary) of adult catfish using the Sigma TRI-reagent method. RT was carried out with 5 μg of total RNA using oligo d(T)₁₈ by superscript-III (Invitrogen) at 50°C in a thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR amplification was done at 94°C (1 min), 58°C (30 s), and 72°C (1 min) for 30 cycles using *sox9aF*-, *sox9aR*-,

sox9bF, and *sox9bR*-specific primers (Table 1). β -actin was PCR amplified at 94 °C (1 min), 60 °C (30 s), and 72 °C (1 min) for 30 cycles using β -actin-specific primers (Table 1) as an internal control to test the quality of cDNA template.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR as described elsewhere (Kwon *et al.* 2001) was carried out to study the expression patterns of *sox9a* and *sox9b* in adult and developing gonads. For this, total RNA was prepared from adult and developing gonads collected from juvenile catfish at different age groups (50, 75, 100, and 150 dph). RT was performed as mentioned above. PCR amplification was done using a thermal cycler (Applied Biosystems) under the following conditions: 94 °C (1 min), 60 °C (30 s), and 72 °C (1 min) for 30 cycles using *sox9a*- and *sox9b*-specific primers (Table 1). Catfish β -actin was PCR amplified at 94 °C (1 min), 60 °C (30 s), and 72 °C (1 min) for 28 cycles using β -actin-specific primers (Table 1) as an internal control.

Real-time quantitative RT-PCR

Expression of *sox9a* and *sox9b* was analyzed by relative qRT-PCR using SYBR Green detection method. Total RNA was extracted from the adult testis and ovary samples, which were collected during four different phases (preparatory, pre-spawning, spawning, and post-spawning phases) of reproductive cycle of catfish using the Sigma TRI-reagent method. RT was carried out with 1 μ g total RNA and random hexamer primers using superscript-III (Invitrogen) according to the manufacturer's protocol. Successful RT was confirmed for all samples by performing PCR amplification of β -actin. Real-time-specific primers for *sox9a*, *sox9b*, and β -actin (Table 1) were designed using Primer Express software (Applied Biosystems) such that at least one of the primers spanned the junction of two exons, giving a single cDNA PCR product and precluding amplification of genomic DNA. PCR was then carried out in 25 μ l reaction in triplicate using power SYBR Green PCR mastermix (Applied Biosystems) in a ABI-7500 fast real-time PCR machine (Applied Biosystems) at 95 °C (15 s) and 60 °C (1 min) for 40 cycles according to the manufacturer's protocol. Dissociation curve analysis was performed for each sample to check single amplification. During PCR, fluorescence accumulation resulting from DNA amplification was recorded using the ABI Prism 7000 sequence detection system software (Applied Biosystems). Cycle threshold (C_t) values were obtained from the exponential phase of PCR amplification. We took β -actin as an endogenous control as it did not show any significant change in C_t values in different phases or treatments. The *sox9* expression was normalized against β -actin expression, generating a ΔC_t value ($\Delta C_t = \text{sox9 } C_t - \beta\text{-actin } C_t$). Relative expression was then calculated according to the equation $2^{-\Delta C_t}$.

In vitro culture of testicular slices in L-15 medium

Testes from catfish in preparatory phase were dissected under sterile conditions and kept in ice-cold Leibovitz (L-15) culture medium (Sigma). Testicular slices of 20 μ m thickness were

prepared using McIlwain tissue chopper (vibratome). Testicular slices were individually transferred to tissue culture plates containing 2 ml of L-15 medium supplemented with 10 mM HEPES and antibiotics (penicillin, 100 IU/ml; streptomycin, 0.1 mg/ml). Testicular slices were cultured for 24 h at 20–22 °C in the presence 100 IU/ml of hCG or saline. At each time interval of 0, 6, 12, and 24 h, slices were collected from both hCG- and saline-treated samples, and washed with ice-cold phosphate buffer (pH 7.4), and total RNA was prepared using Sigma TRI-reagent. The expression of *sox9a* was quantified for each time interval using qRT-PCR. Likewise, the effect of 11-KT (Wako Pure Chemical Industries Ltd, Osaka, Japan) on *sox9a* expression was also examined by treating the testicular slices with 100 ng/ml of 11-KT at different time points. For this purpose, 11-KT was first dissolved in absolute ethanol and later reconstituted in the L-15 culture medium. The expression of *sox9a* was then analyzed using qRT-PCR.

Western blot analysis

Western blot analysis was carried out to detect Sox9 protein in catfish gonadal tissue homogenates using polyclonal Sox9 antibody (LifeSpan Biosciences, Seattle, WA, USA) raised against N-terminal region (1–150 amino acids) of human Sox9 protein that showed 88.5 and 78% homology with the N-terminal region of catfish Sox9a and Sox9b proteins respectively. Adult testis and ovary tissues were homogenized in 250 μ l of homogenization buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM dithiothreitol, 0.1% Triton X-100, and 0.1 mM phenylmethylsulfonyl fluoride using a Sigma hand homogenizer. One hundred micrograms of each sample with a pre-stained marker were electrophoresed through a 15% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Pall Life sciences, Port Washington, NY, USA). Membrane was blocked in 5% skimmed milk powder in Tris-buffered saline (TBS) for 1 h at room temperature. After blocking, membrane was washed five times for 10 min each in TBS with 0.1% Tween 20 (TBST) solution, and incubated with 1:1000 dilution of polyclonal human Sox9 antibody at 4 °C for overnight in 0.5% skim milk powder/TBST solution. Blot was washed and incubated with secondary antibody alkaline phosphatase-conjugated goat anti-rabbit IgG (Bangalore Genei, Bengaluru, India) for 1 h. After washing, blot was developed using BCIP-NBT (Bangalore Genei).

Immunohistochemistry

Both adult and juvenile gonads were fixed in 4% PFA, processed, and embedded in paraplast (Sigma). Sections were cut at 3 μ m thickness using a microtome (Leica, Wetzlar, Germany). Testis and ovary sections were deparaffinized in xylene, rehydrated in successively lower graded concentrations of ethanol, and then treated with 0.1% H_2O_2 for 15 min to prevent endogenous peroxidase reaction. The sections were washed in 0.1 M PBS and then blocked with 10% normal goat serum in PBS for 30 min at room temperature. Sections were then incubated overnight at 4 °C in 1:200 dilution of human SOX9 polyclonal antibody or anti-Sox9 pre-absorbed with excess of synthetic peptide for overnight. Later sections were

washed in PBS with 0.1% Tween 20 (PBST) for 10 min and then incubated with 1:500 diluted biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) at room temperature for 1 h. The sections were incubated with avidin-biotinylated HRP complex (ABC) reagent provided in VECTASTAIN Elite ABC kit (Vector Laboratories) for 30 min at room temperature. The sections were washed in PBS, and color was developed using commercially supplied 3,3'-diaminobenzidine as chromogen and H₂O₂ as substrate for HRP (Vector Laboratories). The sections were washed, dehydrated in graded ethanol, cleared in xylene, and mounted using DPX mountant. Photomicrographs were taken using Olympus CX41 bright field microscope (Olympus, Tokyo, Japan).

Whole-mount IHC

Whole-mount IHC was performed according to the procedure reported earlier (Wang *et al.* 2006) with a few modifications for the detection of Sox9 protein in the ovary of juvenile and mature catfish. Gonads were fixed overnight at 4 °C in 4% PFA, and dehydrated through a methanol series in PBS and kept in –80 °C for a few days. Samples were rehydrated through a reverse methanol series, and blocked with PBS containing 3% skimmed milk powder and 0.1% Triton X-100 (PBMT) overnight at 4 °C. Samples were then incubated overnight at 4 °C in PBMT containing 2% BSA and 1:200 dilution of polyclonal Sox9 antibody, followed by 15 min washes in PBST (0.1% Triton X-100/PBS) for five times at 4 °C. As a negative control, Sox9 antibody pre-incubated with excess of Sox9 synthetic peptide overnight at 4 °C was used as primary antibody. Primary antibody was detected with 1:500 dilution of alkaline phosphatase-conjugated anti-rabbit secondary antibody (Vector Laboratories) in PBMT overnight at 4 °C followed by PBST washes. For enzyme detection, samples were incubated with BCIP–NBT in NTMT buffer (0.1 M NaCl, 0.1 M Tris–HCl, pH 9.5, 50 mM MgCl₂, and 0.1% Tween 20) for 15–30 min. After optimal color development, the reaction was stopped by a PBST wash.

Statistical analysis

All the results were expressed as mean $\pm$ S.E.M. Differences between groups were analyzed for statistical significance by one-way ANOVA followed by Student–Newman–Keuls *post hoc* test using SigmaPlot 9.0 software. A probability of $P < 0.05$ is considered statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

A Grant-in-Aid from University Grants Commission (UGC) (F. No. 34-412/2008), India, to B Senthilkumaran supported this work completely.

Acknowledgements

K Raghuveer acknowledges Council of Scientific and Industrial Research for his senior research fellowship. We thank our Vice-Chancellor, Prof. Seyed E Hasnain, and the Dean, Prof. M Ramanadham, for allowing us to use the Genomics and Microarray facility of School of Life Sciences (SLS), University of Hyderabad. We also thank the support from UGC Centre for Advanced Studies programme for the SLS.

References

- Amores A, Force A, Yan YL, Wang YL, Fritz A, Amemiya C, Lynch M, Prince V, Ho R, Ekker M *et al.* 1998 Zebrafish hox clusters and vertebrate genome evolution. *Science* **282** 1711–1714. (doi:10.1126/science.282.5394.1711)
- Barrionuevo F, Taketo MM, Scherer G & Kispert A 2006 *Sox9* is required for notochord maintenance in mice. *Developmental Biology* **295** 128–140. (doi:10.1016/j.ydbio.2006.03.014)
- Bi W, Deng JM, Zhang Z, Behringer RR & de Crombrughe B 1999 *Sox9* is required for cartilage formation. *Nature Genetics* **22** 85–89. (doi:10.1038/8792)
- Bishop CE, Whitworth DJ, Qin Y, AgoulNIK AI, AgoulNIK IU, Harrison WR, Behringer RR & Overbeek PA 2000 A transgenic insertion upstream of *Sox9* is associated with dominant XX sex reversal in the mouse. *Nature Genetics* **26** 490–494. (doi:10.1038/82652)
- Chen J & Heckert LL 2001 *Dmrt1* expression is regulated by follicle-stimulating hormone and phorbol esters in postnatal Sertoli cells. *Endocrinology* **142** 1167–1178. (doi:10.1210/en.142.3.1167)
- Chiang EF, Pai CI, Wyatt M, Yan YL, Postlethwait J & Chung B 2001 Two *sox9* genes on duplicated zebrafish chromosomes, expression of similar transcription activators in distinct sites. *Developmental Biology* **231** 149–163. (doi:10.1006/dbio.2000.0129)
- Choudhary B, Ganesh S & Raman R 2000 Evolutionary conservation of the gene *Cvsox9* in the lizard, *Calotes versicolor*, and its expression during gonadal differentiation. *Development Genes and Evolution* **210** 250–257. (doi:10.1007/s004270050311)
- Cresko WA, Yan YL, Baltrus DA, Amores A, Singer A, Rodriguez-Mari A & Postlethwait JH 2003 Genome duplication, subfunction partitioning, and lineage divergence: *Sox9* in stickleback and zebrafish. *Developmental Dynamics* **228** 480–489. (doi:10.1002/dvdy.10424)
- Du QY, Wang FY, Hua HY & Chang ZJ 2007 Cloning and study of adult-tissue-specific expression of *Sox9* in *Cyprinus carpio*. *Journal of Genetics* **86** 85–91. (doi:10.1007/s12041-007-0013-z)
- Foster JW, Dominguez-Steglich JA, Guioli S, Kwok C, Weller PA, Stevanovic M, Weissenbach J, Mansour S, Young ID, Goodfellow PN *et al.* 1994 Campomelic dysplasia and autosomal sex reversal caused by mutations in an *SRY*-related gene. *Nature* **372** 525–530. (doi:10.1038/372525a0)
- Guo Y, Cheng H, Huang X, Gao S, Yu H & Zhou R 2005 Gene structure, multiple alternative splicing, and expression in gonads of zebrafish *Dmrt1*. *Biochemical and Biophysical Research Communications* **330** 950–957. (doi:10.1016/j.bbrc.2005.03.066)
- Kent J, Wheatley SC, Andrews JE, Sinclair AH & Koopman P 1996 A male-specific role for *SOX9* in vertebrate sex determination. *Development* **122** 2813–2822.
- Klüver N, Kondo M, Herpin A, Mitani H & Scharf M 2005 Divergent expression patterns of *Sox9* duplicates in teleosts indicate a lineage specific subfunctionalization. *Development Genes and Evolution* **215** 297–305. (doi:10.1007/s00427-005-0477-x)
- Kobayashi T, Kajiura-Kobayashi H, Guan G & Nagahama Y 2008 Sexual dimorphic expression of *DMRT1* and *Sox9a* during gonadal differentiation and hormone-induced sex reversal in the teleost fish Nile tilapia (*Oreochromis niloticus*). *Developmental Dynamics* **237** 297–306. (doi:10.1002/dvdy.21409)

- Koopman P, Schepers G, Brenner S & Venkatesh B 2004 Origin and diversity of the *Sox* transcription factor gene family genome-wide analysis in *Fugu rubripes*. *Gene* **328** 177–186. (doi:10.1016/j.gene.2003.12.008)
- Kwon JY, McAndrew BJ & Penman DJ 2001 Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile tilapia *Oreochromis niloticus*. *Molecular Reproduction and Development* **59** 359–370. (doi:10.1002/mrd.1042)
- Lynch M & Force A 2000 The probability of duplicate gene preservation by subfunctionalization. *Genetics* **154** 459–473.
- Morais da Silva S, Hacker A, Harley V, Goodfellow P, Swain A & Lovell-Badge R 1996 *Sox9* expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. *Nature Genetics* **14** 62–68. (doi:10.1038/ng0996-62)
- Moreno-Mendoza N, Harley VR & Merchant-Larios H 1999 Differential expression of *SOX9* in gonads of the sea turtle *Lepidochelys olivacea* at male- or female-promoting temperatures. *Journal of Experimental Zoology* **284A** 705–710. (doi:10.1002/(SICI)1097-010X(19991101)284:6<705::AID-JEZ12>3.0.CO;2-1)
- Nakamoto M, Suzuki A, Matsuda M, Nagahama Y & Shibata N 2005 Testicular type *Sox9* is not involved in sex determination but might be in the development of testicular structures in the medaka, *Oryzias latipes*. *Biochemical and Biophysical Research Communications* **333** 729–736. (doi:10.1016/j.bbrc.2005.05.158)
- Piera-Velazquez S, Hawkins DF, Whitecavage MK, Colter DC, Stokes DG & Jimenez SA 2007 Regulation of the human *SOX9* promoter by Sp1 and CREB. *Experimental Cell Research* **313** 1069–1079. (doi:10.1016/j.yexcr.2007.01.001)
- Postlethwait J, Amores A, Cresko W, Singer A & Yan YL 2004 Subfunctional partitioning, the teleost radiation and the annotation of the human genome. *Trends in Genetics* **20** 481–490. (doi:10.1016/j.tig.2004.08.001)
- Qin Y & Bishop CE 2005 *Sox9* is sufficient for functional testis development producing fertile male mice in the absence of *Sry*. *Human Molecular Genetics* **14** 221–229. (doi:10.1093/hmg/ddi133)
- Raghuveer K & Senthilkumaran B 2009 Identification of multiple *dmrt1s* in catfish: localization, dimorphic expression pattern, changes during testicular cycle and after methyltestosterone treatment. *Journal of Molecular Endocrinology* **42** 437–448. (doi:10.1677/JME-09-0011)
- Sassone-Corsi P 1995 Transcription factors responsive to cAMP. *Annual Review of Cell and Developmental Biology* **11** 355–377. (doi:10.1146/annurev.cb.11.110195.002035)
- Sekido R & Lovell-Badge R 2008 Sex determination involves synergistic action of *SRY* and *SFI* on a specific *Sox9* enhancer. *Nature* **453** 930–934. (doi:10.1038/nature06944)
- Sekido R, Bar I, Narvaez V, Penny G & Lovell-Badge R 2004 *SOX9* is up regulated by the transient expression of *SRY* specifically in Sertoli cell precursors. *Developmental Biology* **274** 271–279. (doi:10.1016/j.ydbio.2004.07.011)
- Shin HS, An KW, Park MS, Jeong MH & Choi CY 2009 Quantitative mRNA expression of *sox3* and *DMRT1* during sex reversal, and expression profiles after *GnRH*a administration in black porgy, *Acanthopagrus schlegelii*. *Comparative Biochemistry and Physiology* **154** 150–156. (doi:10.1016/j.cbpb.2009.05.013)
- Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischau AM, Lovell-Badge R & Goodfellow PN 1990 A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* **346** 240–244. (doi:10.1038/346240a0)
- Skinner MK 1991 Cell–cell interactions in the testis. *Endocrine Reviews* **12** 45–77. (doi:10.1210/edrv-12-1-45)
- Spotila LD, Spotila JR & Hall SE 1998 Sequence and expression analysis of *WT1* and *Sox9* in the red-eared slider turtle, *Trachemys scripta*. *Journal of Experimental Zoology* **281** 417–427. (doi:10.1002/(SICI)1097-010X(19980801)281:5<417::AID-JEZ7>3.0.CO;2-R)
- Swapna I, Rajasekhar M, Supriya A, Raghuveer K, Sreenivasulu G, Rasheeda MK, Majumdar KC, Kagawa H, Tanaka H, Dutta-Gupta A *et al.* 2006 Thiourea-induced thyroid hormone depletion impairs testicular recrudescence in the air-breathing catfish, *Clarias gariepinus*. *Comparative Biochemistry and Physiology* **144** 1–10. (doi:10.1016/j.cbpa.2006.01.017)
- Takamatsu N, Kanda H, Ito M, Yamashita A, Yamashita S & Shiba T 1997 Rainbow trout *SOX9*, cDNA cloning, gene structure and expression. *Gene* **202** 167–170. (doi:10.1016/S0378-1119(97)00483-6)
- Takase M, Noguchi S & Nakamura M 2000 Two *Sox9* messenger RNA isoforms, isolation of cDNAs and their expression during gonadal development in the frog *Rana rugosa*. *FEBS Letters* **466** 249–254. (doi:10.1016/S0014-5793(00)01078-4)
- Thomsen MK, Butler CM, Shen MM & Swain A 2008 *Sox9* is required for prostate development. *Developmental Biology* **316** 302–311. (doi:10.1016/j.ydbio.2008.01.030)
- Verhoeven G, Willems A, Denolet E, Swinnen JV & De Gendt K 2010 Androgens and spermatogenesis: lessons from transgenic mouse models. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **365** 1537–1556. (doi:10.1098/rstb.2009.0117)
- Vidal VP, Chaboissier MC & De Rooij DG 2001 *Sox9* induces testis development in XX transgenic mice. *Nature Genetics* **28** 216–217. (doi:10.1038/90046)
- Wade MG & Van der Kraak G 1991 The control of testicular androgen production in the goldfish: effects of activators of different intracellular signalling pathways. *General and Comparative Endocrinology* **83** 337–344. (doi:10.1016/0016-6480(91)90138-V)
- Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, Pasantes J, Bricarelli FD, Keutel J, Hustert E *et al.* 1994 Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the *SRY*-related gene *SOX9*. *Cell* **79** 1111–1120. (doi:10.1016/0092-8674(94)90041-8)
- Wang Y, Risteovski S & Harley VR 2006 *SOX13* exhibits a distinct spatial and temporal expression pattern during chondrogenesis, neurogenesis, and limb development. *Journal of Histochemistry and Cytochemistry* **54** 1327–1333. (doi:10.1369/jhc.6A6923.2006)
- Western PS, Harry JL, Graves JAM & Sinclair AH 1999 Temperature dependent sex determination, upregulation of *Sox9* expression after commitment to male development. *Developmental Dynamics* **214** 171–177. (doi:10.1002/(SICI)1097-0177(199903)214:3<171::AID-AJA1>3.0.CO;2-S)
- Xia W, Zhou L, Yao B, Li CJ & Gui JF 2007 Differential and spermatogenic cell-specific expression of *DMRT1* during sex reversal in protogynous hermaphroditic groupers. *Molecular and Cellular Endocrinology* **263** 156–172. (doi:10.1016/j.mce.2006.09.014)
- Yan YL, Miller CT, Nissen RM, Singer A, Liu D, Kirn A, Draper B, Willoughby J, Morcos PA, Amsterdam A *et al.* 2002 A zebrafish *Sox9* gene required for cartilage morphogenesis. *Development* **129** 5065–5079.
- Yokoi H, Kobayashi T, Tanaka M, Nagahama Y, Wakamatsu Y, Takeda H, Araki K, Morohashi K & Ozato K 2002 *Sox9* in a Teleost Fish, Medaka (*Oryzias latipes*): evidence for diversified function of *Sox9* in gonad differentiation. *Molecular Reproduction and Development* **63** 5–16. (doi:10.1002/mrd.10169)
- Zhou R, Liu L, Guo Y, Yu H, Cheng H, Huang X, Tiersch TR & Berta P 2003 Similar gene structure of two *Sox9a* genes and their expression patterns during gonadal differentiation in a teleost fish, rice field eel (*Monopterus albus*). *Molecular Reproduction and Development* **66** 211–217. (doi:10.1002/mrd.10271)

Received 27 April 2010

First decision 1 June 2010

Accepted 28 June 2010