Involvement of *pax2* in ovarian development and recrudescence of catfish: a role in steroidogenesis

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Abstract

PAX2, a member of paired box family, is an essential transcription factor for the organ development in vertebrates including teleosts, yet no evidence has been shown for its involvement in reproduction. To study this, partial- and/or full-length cDNA of *pax2* was isolated from the ovary of catfish, *Clarias batrachus*, along with its other Pax family members, *pax1* and *pax9*. Tissue distribution and ontogeny expression analysis indicated the prevalence of *pax2* but not *pax1* and *pax9* in ovary. Varied phase-wise expression during ovarian cycle and elevation of *pax2* after human chorionic gonadotropin induction showed probable regulation by gonadotropins. Pax2 could be localized in various stages of oocytes and in follicular layer of vitellogenic and post-vitellogenic oocytes. To assess the functional significance of *pax2*, transient RNA silencing was performed using primary catfish ovarian follicle culture, *in vitro*, and in catfish, *in vivo*, through ovary-targeted injection of PEI-esiRNA. Pax2 siRNA treatment reduced the expression of various transcripts related to ovarian development like signaling molecules such as *wnt4* and *wnt5*, estrogen receptors, several steroidogenic enzymes and transcription factors. These transitions in transcript levels might have been mediated by Pax2 acting upstream of *wnt4/5* that may play a role in steroidogenesis and/or ovarian development along with *ad4bp/sf-1* or by direct or indirect interaction with steroidogenic enzyme genes, which is evident from the change in the levels of serum estradiol-17 β but not 17 α ,20 β -dihydroxy-4-pregnen-3-one. Taken together, it seems that *pax2* has a plausible role during ovarian development and/or recrudescence of catfish either directly or indirectly through Wnt signaling pathway.

Key Words

- ▶ ovarian follicles
- ▶ paired box
- ▶ Wnt signaling
- ▶ steroidogenesis

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Introduction

Paired box (PAX) genes encode a family of transcription factors that are involved in the development of various tissues during early embryogenesis in vertebrates. They are known to possess paired domain and derived their name by being homologous to the *Drosophila* gene, 'prd' that is involved in the pair-rule

segmentation (Treisman *et al.* 1991). There are nine Pax genes identified in humans that are divided into four subgroups based on the presence or absence of a homeodomain and an octapeptide region, and their developmental functions are assigned based on the subgroup they belong to (Blake & Ziman 2014).

Pax2 is known to have a role in mammalian kidney development (Torres *et al.* 1995), inner ear patterning and optic nerve trajectory (Torres *et al.* 1996) and also operates various signaling pathways (Zhou 2012).

Group of *pax* genes identified till date in teleosts are known to be involved in various developmental processes. Hindbrain development in teleosts is dependent on *pax1* and *pax9*, whereas *pax6* plays a role in retinal development (Lakowski *et al.* 2007). Myogenesis is mediated by the expression of *pax3* and *pax7* (Kacperczyk *et al.* 2009, Froehlich *et al.* 2013, Chapalamadugu *et al.* 2015), whereas *pax8* is an early marker for inner ear growth and is also required for otic placode development (Mackereth *et al.* 2005). For instance, *pax2*, in teleosts, plays an important role in several developmental processes such as retinal regeneration in the optic nerve head of goldfish (Parrilla *et al.* 2012). In zebrafish, it maintains the otic placode development in the inner ear along with *pax8* (Mackereth *et al.* 2005). It is also required for the development of mid-hind brain boundary (Lun & Brand 1998), kidney (Majumdar *et al.* 2000) and thyroid follicles in zebrafish (Wendl *et al.* 2002). Considering the interaction and regulatory influence of thyroid hormones on gonadal development and recrudescence (Swapna *et al.* 2006, Swapna & Senthilkumaran 2007), the importance of *pax* genes in gonads needs to be explored. Further, the direct role of these Pax members in gonadal development, if any, has not yet been analyzed in lower vertebrates.

Catfish, *Clarias batrachus*, is an annual breeder that follows four reproductive phases, viz. preparatory (February–April), pre-spawning (May–June), spawning (July–August) and post-spawning/resting phase

(September–January). During the spawning phase, the membrane-bound bi-lobed ovary consists of a large number of post-vitellogenic follicles (Lehri 1968), which can be ovulated artificially for *in vitro* fertilization with testicular milt. The ovary undergoes morphological and physiological changes during annual reproductive cycle as per seasonal cues. Depending on the stage of ovary, the expression pattern of various transcripts undergoes changes in teleosts. In view of this, an attempt has been made to investigate the role of Pax2 in ovarian development during the reproductive cycle of catfish, primarily through expression analysis and localization and after gonadotropin induction. cDNA fragments of *pax2*, *pax1* and *pax9* were cloned either partially or full-length to determine the tissue distribution to evaluate gonad specificity in comparison with *pax2*. Further, to assess the functional significance of *pax2* expression in ovarian development, transient siRNA transfection strategy was used to silence the gene expression, *in vitro* and *in vivo*. *In vitro* transfection was done on primary catfish ovarian follicle culture by the most common approach used for nucleic acid delivery, with the lipid-based molecule, Lipofectamine, as the transfection reagent. *In vivo* transfection into animal models is limited by various barriers. Of the various methods used to efficiently silence the target gene with less off-targets, the non-viral delivery method of polyethylenimine (PEI) transfection, known to be effective for even therapeutic applications (Akhtar & Benter 2007), was chosen. After transient silencing of mRNA and protein levels of Pax2, various factors that are known to play a critical role in ovarian development of

Table 1 List of primers used for cDNA cloning.

Sl. no.	Primer	Sequence (5'–3')	Usage
1	P2 DF*	GTTCNTCYATHAACAGGAT	RT-PCR
2	P2 DR*	TCDCYCCAGGVACCATTC	RT-PCR
3	P1 DF*	CCSCTKCCCAAYGCSATC	RT-PCR
4	P1 DR*	ACRTTRTAYTTRTCRCVAC	RT-PCR
5	P1 5P	TCGCACCGGGCAATATGGAGCCGGT	RACE
6	P15N	ACGGATCCCGAGCTGTGCTAACTCCA	RACE
7	P13P	AGGCAGCAAGCCGCGAGTTACGACACCA	RACE
8	P13N	TGCATGGGAGATTCTGTGACCGACTTC	RACE
9	P25P	CCGCTCGATACTGGAGGGGAAGCTGTGC	RACE
10	P25N	TCCATCAGGAGAGGGGTGGTACTGTG	RACE
11	P23P	GCCTTGGATCGAGTGTGTTGAGCGACC	RACE
12	P23N	CAACCTTACCTGGGTATCCTCCTCGT	RACE
13	P2 Orf fw	ATGGATATTTCACTGCAAAGCAGAC	ORF cloning
14	P2 Orf rv	CTAGTGGCGGTCATAGGCA	ORF cloning

*IUPAC nucleic acid codes are B=C or G or T; D=A or G or T; H=A or C or T; K=G or T; M=A or C; N=any base; R=A or G; S=G or C; V=A or C or G; W=A or T; Y=C or T.

catfish were analyzed. To this end, transcription factors such as steroidogenic factor 1 (*ad4bp/sf-1*), forkhead box L2 (*foxl2*), SRY-related high mobility group (HMG) box family member, 9b (*sox9b*) and estrogen receptor 1 (*esr1*) and 2 (*esr2*), which are essential for ovarian development (see Sirotkin 2010, Sudhakumari & Senthilkumaran 2013) were chosen for analysis along with the signaling molecules of *wnt* (wingless-integrated (MMTV) site family) 4 and 5 (*wnt4* and *wnt5*). In addition, serum estradiol-17 β (E_2) and 17 α ,20 β -dihydroxy-4-pregnen-3-one (17 α ,20 β -DP) were measured as the production of these steroids in the ovary and their subsequent signaling events are critical for proper ovarian developmental processes of follicular growth, oocyte maturation and ovulation through shift in steroidogenesis (Senthilkumaran *et al.* 2004, Drummond 2006, Jamnongjit & Hammes 2006). Further, the expression patterns of several transcripts encoding steroidogenic enzyme genes, such as cholesterol side-chain cleavage enzyme of cytochrome P450 family member 11 subfamily A member 1 (*cyp11a1*), 3 β -hydroxysteroid dehydrogenase (*3 β -hsd*), 17 β -hydroxysteroid dehydrogenase type 1 and 12 (*17 β -hsd1* and *12*), ovarian aromatase (*cyp19a1a*), 17 α -hydroxylase/C17–20 lyase type 1 (*cyp17a1*), 20 β -hydroxysteroid dehydrogenase (*20 β -hsd*) and steroidogenic acute regulatory protein (*star*), were analyzed.

Materials and methods

Animals and sampling

The catfish *C. batrachus* fingerlings and adults used in this study were obtained by *in vitro* fertilization established in our laboratory (Rajakumar *et al.* 2012) at Hyderabad with the brood stock procured from local markets of river Ganges of Varanasi, India. Catfish rearing, feeding and maintenance were done as described earlier (Rajakumar *et al.* 2012). For tissue distribution study, different tissues (ovary, brain, liver, kidney, gills, intestine, muscle, heart and spleen) from adult female ($n=6$) and male (testis) catfishes ($n=6$) were collected during preparatory phase of reproductive cycle. For ontogeny analysis, 0 days post hatch (dph), mesonephric gonadal complex (MGC) from 5, 10, 30 and 40dph larvae ($n=15$) and gonads ($n=15$) from the sexual differentiation stage of 50dph till adult (50, 100, 150, 200, 250 and 365dph) were collected separately from males and females. As per the seasonal reproductive cycle characterized histologically in our laboratory, ovaries and testes were collected ($n=10$) at different months for phase-wise analysis. All the tissues were stored at -80°C until total RNA preparation. Animal maintenance, experimentation and killing were done following the general institutional animal ethical guidelines, University of Hyderabad.

Table 2 List of primers used for qPCR analysis.

Gene name/symbol	Forward primer (5'–3')	Reverse primer (5'–3')	Accession number
<i>pax2</i>	ATCCTGGGCAGGTACTATGA	GGATCTCCACGCGAACAT	KU301794
<i>pax1</i>	ACCGGCTCCATATTGCCCG	TATCGCACACTCCATCTGCT	KX499463
<i>pax9</i>	CAGCTCGATTAGTCGGATCCT	GTACTTTAGTCCCTGTGGCTG	KX499464
<i>wnt4</i>	AGCAACTGGCTCTATCTGG	ACTCGTCGATGGCAAGCTG	KX499465
<i>wnt5</i>	TCATGGTGGTCTCTGGCTATG	ATGTACTGCATGTGGTCCTG	KX529112
<i>foxl2</i>	CATGGCTATACGCGACAGCTC	CCAGTAGTCCCCTTCCTCTC	HQ680981
<i>sox9b</i>	GAGACCCAGTCAGGCCACAG	AGGGTCTCGATGTGGGCCA	HM149259
<i>star</i>	TCGTCCGAGCCGAGAACGG	TGCCTCCTCACTCCACTG	FJ793811
<i>ad4bp/sf-1</i>	TCACTATGGCCTGCTCACCTG	CGCTTGATACATGGGGCCGAAC	HQ680985
<i>cyp17a1</i>	CCATGGCTCCAGCTCTTTCC	CAGTAAGACCAACATCCTGAGTGC	FJ790422
<i>cyp11a1</i>	TCAACCAAGCGGACCACTGT	TCAGGATGCCGTGCCAACTC	KF739411
<i>17β-hsd1</i>	GACATCTGGTGTGTAATGCAGG	CTGCCGTGTGACCAGGATCCGT	HQ680984
<i>17β-hsd12</i>	AGCCATCGAGAGCAAGTACCATGT	AAGCCGAGTCATCTGACAAACCGA	JN848590
<i>20β-hsd</i>	GAATCGCCTTCAAAATGG CTG	AACATTCACCACCCTTCCTCC	FJ031008
<i>3β-hsd</i>	GAGGTAAATGTGAAAGGTACCAA	TAGTACACAGTGTCTCATGG	HQ680983
<i>cyp19a1a</i>	AGGTCCCTGGTTTGTCTG	TGCAGATGGCCTGCTGAGG	GU220075
<i>esr1</i>	TGTCGGGCCACCAATCA	GTGTCTCTGCTGTGCTTCAT	X84743
<i>esr2</i>	TGTCCTGCAACCAACCA	CCTCTGCTGAGACCCCCACGA	Teves <i>et al.</i> (2003)*
<i>18S rRNA</i>	GCTACCACATCCAAGGAAGGCAGC	CGGCTGCTGGCACCAGACTTG	KM018296

*No accession number available.

Molecular cloning of different cDNA fragments of *Pax* from catfish

Adult catfish ovary (100mg) was used to isolate total RNA using TRI reagent (Sigma). The quantity was analyzed by NanoDrop spectrophotometer (ND-2000, NanoDrop Technologies, Wilmington, DE, USA) and its 260/280 ratio was obtained as a basis to ensure its purity. To check its integrity, denaturing gel electrophoresis was performed, which showed two sharp and clear bands of 28S and 18S rRNA in approximately 2:1 ratio without smear, indicating intact RNA. The cDNA was prepared with SuperScript III according to the manufacturer's instructions (Invitrogen), followed by DNase I treatment to exclude DNA contamination, using 5µg of total RNA after checking its quality by amplifying the β -actin gene. To isolate partial cDNA fragments of *pax1* and *pax2*, degenerate primers were designed (Table 1) based on the available nucleotide sequences of teleosts and other vertebrates from NCBI GenBank database. Partial fragments of *pax1* (~250bp) and *pax2* (~620bp) obtained from the PCR amplifications performed at 94°C (1min) and 35 cycles of 94°C (30s), 53°C (30s), 72°C (1min) and 72°C (10min) using respective degenerate primers (P2DF, P2DR and P1DF, P1DR) listed in Table 1 were cloned into the pGEM-T easy vector (Promega) and sequenced bidirectionally. Gene-specific primers (Table 1) were designed based on the sequence information of the partial cDNAs to obtain full-length cDNA through RACE strategy (Clontech). All nested PCR fragments cloned into the pGEM-T easy vector (Promega) after gel purification were sequenced bidirectionally. Interestingly, cDNA fragment of *pax9* was amplified while performing 3' RACE for *pax1*. All overlapping DNA fragments were assembled, and the full-length cDNA sequencing data were analyzed using Lasergene software 7.1.0 (DNASTAR, Madison, WI, USA) and verified using the NCBI-BLAST and ClustalW2 under default parameters.

Sequence and phylogenetic analysis

Pax2 amino acid (aa) sequences of other vertebrates from GenBank were used for the ClustalW alignment and

phylogenetic analysis with the deduced aa sequence of catfish Pax2. The GenBank accession numbers of sequences used are *Danio rerio* Pax2a (NP_571259.1), *D. rerio* Pax2b (NP_571715.1), *Dicentrarchus labrax* (CBN81534.1), *Oryzias latipes* (CAB09696.1), *Ovis aries* (NP_001171523.1), *Gallus gallus* (NP_990124.1), *Mus musculus* (NP_035167.4), *Rattus norvegicus* (NP_001099831.1), *Xenopus laevis* (CAA71205.1), *Homo sapiens* (NP_000269.2) and *C. batrachus* (KU301794). ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) was used for the multiple alignment. Jalview 2.8. ClustalW (<http://clustalw.ddbj.nig.ac.jp/>) was used to construct the phylogenetic tree by neighbor-joining method, and it was displayed using TreeView 1.6.6. The values represent bootstrap scores out of 1000 trials, indicating the credibility of each branch.

Quantitative real-time PCR (qPCR)

One microgram of total RNA, extracted using TRI reagent (Sigma) as per the manufacturer's instructions, was used with random hexamers for the reverse transcription using Verso Reverse Transcriptase (Thermo Scientific), followed by DNase I treatment to exclude genomic DNA. For all qPCR analyses, specific primers of all transcripts including *wnt4* and *wnt5* cloned from our laboratory (Table 2) were designed for the amplicon length of ~150–250bp with at least one primer to span the exon–exon junction with the existing fish and mammalian genome information as basis. To check the specificity and efficiency of the primers used, semi-quantitative PCR was performed before proceeding to qPCR, and the PCR product was sequenced. All reactions were performed in triplicates for three different samples with Power SYBR Green PCR Master Mix (Applied Biosystems) in an ABI Prism 7500 fast thermal cycler (Applied Biosystems) according to the manufacturer's universal thermal cycling conditions. After performing melting-curve analysis to check the specificity of the PCR amplification, cycle threshold (Ct) values obtained from the exponential phase of PCR amplification were used for generating Δ Ct value, in which 18S rRNA is taken for normalization against the expression of target gene. From the preliminary experiments of this study, as

Figure 1

Relative mRNA levels of (A) *pax1*, (B) *pax9* and (C) *pax2* in different tissues of adult catfish. All data were expressed as mean $\pm$ S.E.M. Means with different letters differ significantly ($P < 0.05$; Kruskal–Wallis one-way ANOVA on ranks followed by SNK test). T, Testes; O, Ovary; B, Brain; G, Gill; H, Heart; K, Kidney; L, Liver; I, Intestine; S, Spleen; M, Muscle.

per the validation done based on Radonić *et al.* (2004), 18S rRNA (KM018296.1) normalization was found to be efficient and constitutive with the lowest transcription range compared with other reference genes such as β -actin (EU527190.2) and *gapdh* (KC414932.1). The evaluation of gene expression was done by $2^{-\Delta Ct}$ method.

Human chorionic gonadotropin (hCG) induction, *in vivo*

Acclimated adult female catfishes ($n=10$) used for this experiment were injected with a single dose (1000IU/kg body weight) of hCG (Pubergen, Uni-Sankyo Pvt. Ltd., Hyderabad, India) for the induction, whereas control fishes were administered with physiological saline (0.6% NaCl W/V) during mid-preparatory phase (March). The ovaries were collected separately for saline- and hCG-induced fishes at different time intervals (0, 6, 12, 18 and 24h) after killing them by anesthetizing in mild ice-cold water having MS 222 (Sigma). The relative expression of *pax2* was analyzed using qPCR as described previously.

In situ hybridization (ISH)

Ovary (100 mg) of adult catfish ($n=10$) in pre-spawning phase was fixed in 4% PFA in PBS (phosphate buffered saline, pH 7.4) at 4°C overnight. PFA-fixed ovary was washed four times with PBS for a cycle of 10 min each at 4°C. Embedding was done in OCT compound medium (Leica Microsystems) onto a cryomold (Tissue-Tek,

Sakura Finetek Europe B.V., AJ Alphen aan den Rijn, The Netherlands) and stored at -80°C until sectioning. The OCT-embedded tissues were cross-sectioned (10 μm) on to poly-L-Lysine coated slides using a cryostat (Leica CM1850, Leica Microsystems) and were allowed to dry overnight on a hotplate at 42°C . The *pax2* probe was prepared from pGEM-T easy-*pax2* cDNA (~620bp) by linearization using restriction enzymes (zero cutters for insert). Based on the sequence data of pGEM-T-*pax2* cDNA, either T7 or SP6 RNA polymerase was used for sense and antisense 'cRNA' probe preparation using digoxigenin (DIG; Roche Diagnostics GmbH) -based *in vitro* transcription. ISH protocol was performed as per the method described in Rajakumar and Senthikumar (2014a).

Western blot analysis

The antibodies used to carry out western blot analyses are Pax2 (Life Span Biosciences Inc. LS-C102856)/Wnt4 (Life Span Biosciences Inc. LS-C109642/61282) polyclonal antibodies raised against the conserved C-terminal regions of human Pax2 and Wnt4, which showed 80% homology with the conserved region of catfish Pax2 and Wnt4, respectively. Ovarian tissue homogenate was extracted from adult catfish ovary with homogenization buffer containing 50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM DTT, and ProteoBlock protease inhibitor cocktail (Thermo Scientific), followed by a standard protocol

Figure 2

Relative mRNA levels of *pax2* at (A) various stages of gonadal development from 0 to 365 days post hatch, (B) different phases of ovarian and testicular reproductive cycle, (C) diverse time intervals after hCG induction in ovary and (D) different developmental stages of ovarian follicles. All data were expressed as mean $\pm$ S.E.M. * indicates means with significantly higher *pax2* mRNA levels compared with the corresponding opposite sex of the same age group ($P < 0.05$; ANOVA followed by SNK test). Means with different letters differ significantly ($P < 0.05$; Kruskal-Wallis one-way ANOVA on ranks followed by SNK test). ** indicates means with significantly higher *pax2* mRNA levels compared with the 0h and parallel control ($P < 0.05$, $P < 0.01$, ANOVA followed by SNK test). M, Male; F, Female; P, Preparatory; PS, Pre-spawning; S, Spawning; R, Post-spawning/Resting Phase. I. Peri-nucleolar, II. Pre-vitellogenic, III. Vitellogenic and IV. Post-vitellogenic oocytes.

for western blot as described earlier in Rajakumar and Senthilkumar (2014a). The blots were developed with commercial enhanced chemiluminescence (ECL) western blotting substrate as per the manufacturer's instructions (Promega). Same protocol was followed for anti- β -tubulin antibody, which was used as an equal loading control. For negative control, muscle or intestine tissue homogenate protein was used.

Immunohistochemistry

Adult catfish ($n=10$) ovary was fixed and sectioned as described previously. The sections were rinsed with PBS, blocked with goat serum and incubated with anti-Pax2 polyclonal antibody (1:1000) or pre-adsorbed antibody (for negative control) overnight at 4°C in a humid chamber. Further, HRP-conjugated secondary antibody (Merck Bangalore Genei) incubation was done for 2 h at RT. VECTASTAIN Elite ABC reagent (Vector Laboratories, Burlingame, CA, USA) incubation was done

for 30 min before developing them with commercially available 3,3'-diaminobenzidine (DAB) and H_2O_2 (Vector Laboratories) as substrate. After developing, sections were counterstained with hematoxylin (Qualigens Fine Chemicals, Worli, Mumbai, India), dehydrated using a graded ethanol series and mounted using DPX mountant. The images were acquired using Q capture Pro 6 software (Quantitative Imaging Corporation Surrey, BC, Canada) with Micropublisher 3.3 RTV-CCD camera in a CX41 Olympus microscope (Olympus Corporation).

Primary catfish ovarian follicle culture (mixed)

Primary ovarian follicle culture was done as per the protocol of Li and coworkers (2012) with minor modifications. Catfish ($n=10$) ovaries in preparatory phase, which are abundant in transcripts essential for reverting to functional stage, were dissected, and the follicles (pre-vitellogenic stage) were dispersed gently using a sterile plastic pipette. The follicular culture has

Figure 3

Localization of mRNA of *pax2* in the adult catfish ovary with (A, C and E) anti-sense probe and (B, D and F) sense probe showing the presence or absence of transcripts in different stages of ovarian follicles. (G) Hematoxylin and eosin staining of ovary showing different types of follicles. I. Peri-nucleolar, II. Pre-vitellogenic, III. Vitellogenic and IV. Post-vitellogenic oocytes. (H) Western blot analysis demonstrating the antibody characteristics of Pax2. Pax2 antibody was detected as a putative ~42 kDa band in kidney and ovary but not in muscle, whereas β -tubulin showed no such differences. (I, K) Localization of Pax2 in the adult catfish ovary using immunohistochemistry. (J, L) No immunoreactive signal was evident in the negative control with pre-adsorbed Pax2 antibody with excess peptide of Pax2. Arrows indicate ovary sections showing the presence or absence of signals. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-16-0103>.

Figure 4

(A) Relative mRNA levels of *pax2*, *wnt4* and *wnt5*. (B) Western blot analysis of Pax2 and control β-tubulin in the primary culture of ovarian follicles for 4 days. C, control. (C) Representative phase-contrast image of primary cultures of catfish ovarian follicles and (D) after control siRNA transfection (to check the efficiency). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-16-0103>.

different cell types and was hence mentioned as mixed. The dispersed follicles ($n=200$) were washed with 60% L-15 medium (Sigma) several times. The filtered follicles were then washed several times with medium M199 (Gibco-BRL, Invitrogen) and plated into 10 cm culture dishes. The follicles were grown for 6 days at 28°C in M199 with 10% FBS (Gibco-BRL) with a change of medium after 3 days. Further, the follicular cells during the 6-day incubation were suspended by trypsinization, washed and sub-cultured into 24-well plates in Dulbecco's Modified Eagle's Medium (DMEM).

siRNA transfection *in vitro*

The primary culture cells (100,000 cells/well) obtained from ovarian follicles were grown in DMEM with 10% FBS, 2 mM L-glutamine, and antibiotic and antimycotic solution (Gibco-BRL) at 37°C supplemented with 5%

CO₂. Transfection was done using Lipofectamine 2000 reagent (Invitrogen) as per manufacturer's protocol. Custom-made catfish-specific MISSION esiRNA (Sigma), an endoribonuclease-prepared heterogeneous mixture of siRNA pools with multiple triggers and less off-target effects, synthesized from the conserved region of Pax2 (250–386 aa; GenBank accession no. KU301794), was used for the experiments. The fluorescent universal negative control esiRNA was purchased from Sigma (Cat. No. SIC007-10NMOL), for control siRNA experiments. These experiments were performed as per the institutional biosafety committee ethical guidelines, University of Hyderabad.

siRNA injection *in vivo*

Adult female catfish ($n=6$) at the preparatory phase (March) used for our experiments were obtained by *in vitro* fertilization followed by rearing and

Figure 5

Relative mRNA levels of *pax2* in the ovary of catfish after silencing of primary culture of follicles (A) at different concentrations after 48 h (B) at 10 ng concentration after different periods. (C) Western blot analysis after silencing Pax2 at 10 ng concentration after different periods. Relative mRNA levels of (D) *wnt4* and (E) *wnt5* in the ovary of catfish after silencing of *pax2* in primary culture of follicles at 10 ng concentration after different periods. (F) Relative mRNA levels of *pax2*, *wnt4* and *wnt5* after transfecting with control siRNA. The relative expression was normalized with 18S rRNA expression, and the values were calculated using comparative ct method. All data were expressed as mean $\pm$ S.E.M. (* $P < 0.05$; ANOVA followed by SNK test).

maintenance in our laboratory as explained earlier. Fish were briefly anesthetized before surgery with 100 mg/L of MS 222 (Sigma) in mild ice-cold water following general animal ethical guidelines. A minor incision was made at the abdomen with the help of sterile scalpel and a syringe loaded with 1 µg of siRNA complexed with linear PEI (Sigma) was injected directly into the ovary bilaterally. The absorption of the PEI-siRNA complex into the ovary was checked with trypan blue for mock control. Then the incision was sutured by catgut under sterile conditions, and fishes were kept in water at ambient temperature for a few minutes to recover from anesthesia. The same procedure was followed for the control siRNA treatment. The injected fish were maintained in different tanks holding water of 50 L capacity with continuous aeration, and replenishment of water was done for the control and treated for 3 days with regular food supply. After 3 days, the fishes were killed for the analysis of their ovarian tissues.

Enzyme immunoassay (EIA) for E₂ and 17α,20β-DP

Levels of E₂ and 17α,20β-DP in serum were measured in the control and after siRNA transfection by EIA by following the manufacturer's (Cayman) protocol. Intra- and inter-assay variations were within the limits specified in the manufacturer's protocol. The sensitivity of detection limit for E₂ and 17α,20β-DP were 20 pg/mL and 4 pg/mL, respectively. The cross-reactivity of the E₂ antisera with estrone is 12%, with estradiol-17-glucuronide is 10% and with estriol is 0.3%. Anti-17α,20β-DP serum is 100% specific with minimal cross-reactivity with 20β-hydroxyprogesterone (0.01%) and 17α,20α-dihydroxy progesterone (0.004%). Assays were performed in triplicates for each independent sample, and serum sample dilutions were in parallel with the standard dilutions.

Statistical analysis

All data are presented as means of different samples with standard error of the mean (S.E.M.). All data either passed normality and homogeneity tests or were compared by Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks, followed by Student-Newman-Keuls' (SNK) *post hoc* test. All statistical analyses were performed using SigmaPlot 11.0 software (Systat Software Inc., Chicago, IL, USA). A probability of $P < 0.05$ was considered statistically significant.

Results

Molecular cloning of different cDNA fragments of Pax from catfish and phylogenetic analysis

Partial cDNA fragment of ~620 bp obtained from catfish was confirmed as *pax2* by NCBI-BLAST search, and the nucleotide sequence data were used to design specific primers for 5' and 3' RACE. After aligning the sequences amplified through 5' and 3' RACE, the ORF obtained was of ~1164 bp encoding ~387 aa with deduced molecular weight of ~42 kDa. The sequences obtained at 5' UTR was of ~534 bp and 3' UTR was of ~626 bp with poly-A⁺ tail.

Figure 6

Relative mRNA levels of (A) *star* and steroidogenic enzymes such as *cyp17a1*, *cyp19a1a*, *17β-hsd1*, *20β-hsd*, *17β-hsd1* and *12*, (B) transcription factors, *foxl2*, *sox9b* and *ad4bp/sf-1* and estrogen receptors, *esr1* and *esr2* in the control and after silencing of primary culture of ovarian follicles, *in vitro*. The relative expression was normalized with *18S rRNA* expression, and the values were calculated using comparative ct method. All data were expressed as mean ± S.E.M. (* $P < 0.05$; ANOVA followed by SNK test). NS, not significant.

The full-length cDNA fragment obtained was of ~2.3 kb and was submitted to GenBank (KU301794). The partial cDNA fragment of ~250bp of *pax1* was used to design specific primers for obtaining full-length fragment of ~1.5 kb with the RACE cDNA fragments of 5' end and 3' end. At the 3' end, two fragments, with homology to *pax1* (~1kb) and *pax9* (~900bp), were obtained and were submitted to GenBank (*pax1* (KX499463) and *pax9* (KX499464)). The deduced aa sequence obtained for Pax2 from catfish showed high homology for the conserved paired box domain (16–141 aa), DNA-binding helix-turn-helix motif (37–139 aa) and C-terminal Pax2 domain (278–386 aa) with other vertebrate species aligned on multiple sequence alignment. The phylogenetic analysis showed that it forms a clade with its counterparts from other teleost species with high similarity (figure not shown).

Expression levels of *pax1*, *pax9* and *pax2* in different tissues of adult catfish

The expression of *pax1* (Fig. 1A) and *pax9* (Fig. 1B) analyzed in different tissues of adult catfish showed ubiquitous nature in all tissues with higher levels in brain. However, *pax2* (Fig. 1C) showed dominance in brain, ovary and kidney in comparison with lower levels observed in other tissues.

Expression levels of *pax2* during gonadal ontogeny and reproductive cycle, after hCG induction and in different stages of ovarian follicles of catfish

The relative expression pattern of *pax2* from 0dph till adult revealed an increase from 0 to 40dph. The expression pattern from 50dph to adult analyzed in both males and females showed significantly higher levels ($P < 0.05$) in ovary compared with testis (Fig. 2A). The analysis of *pax2* expression during different phases of ovarian reproductive cycle of catfish showed its higher levels in preparatory and spawning phases compared with those in other phases (Fig. 2B) and are significantly ($P < 0.05$) high in levels compared with respective testicular phases (Fig. 2B). A single dose of hCG induction to catfish, *in vivo*, significantly increased the expression ($P < 0.05$; $P < 0.01$) pattern at 6, 12 and 18h and decreased at the end period of 24h compared with the parallel controls and at 0h period (Fig. 2C). The relative expression of *pax2* in different stages of the ovarian follicles showed significantly ($P < 0.05$) high levels in peri-nucleolar and pre-vitellogenic oocytes followed by vitellogenic and post-vitellogenic oocytes (Fig. 2D).

Localization of *pax2* mRNA and protein in catfish ovary

Localization of mRNA of *pax2* in the ovary of catfish by ISH revealed its presence in peri-nucleolar, pre-vitellogenic, vitellogenic and follicular layer of vitellogenic and post-vitellogenic oocytes with antisense probe (Fig. 3A, C and E),

Figure 7

Relative mRNA levels of *pax2*, *wnt4* and *wnt5* in the control and (A) after silencing of *pax2*, (B) after silencing with control siRNA, (C) Western blot analysis showing silencing Pax2 and Wnt4 of proteins after silencing with PAX2, in the ovary of catfish, *in vivo*. The relative expression was normalized with *18S rRNA* expression, and the values were calculated using comparative ct method. All data were expressed as mean $\pm$ S.E.M. (* $P < 0.05$; ANOVA followed by SNK test).

whereas sense probe elicited no signal, and it had only the counterstain (Nuclear Fast Red) used (Fig. 3B, D and F). The stages of follicles can be observed clearly with hematoxylin and eosin-stained ovarian sections (Fig. 3G). Immunoreactivity observed for Pax2 in the ovary of catfish with heterologous antibody gave single specific band of Pax2 of ~42 kDa (Fig. 3H) and also showed typical localization pattern like ISH (Fig. 3I and K). Immunoreactivity was completely absent when pre-absorbed Pax2 antibody with excess peptide of Pax2 was used as a control (Fig. 3J and L), which confirmed the antibody specificity.

Silencing of *pax2* in primary culture of catfish ovarian follicles, *in vitro* at mRNA and protein levels

The transcript expression levels observed for *pax2*, *wnt4* and *wnt5* for 4 days of primary culture cells showed a constitutive pattern, before transfection with siRNA of *pax2* (Fig. 4A), and the protein levels of Pax2 and Wnt4 were also found to be stable (Fig. 4B). The transfection efficiency of primary follicle culture cells (Fig. 4C) was analyzed and confirmed with control siRNA transfection (Fig. 4D). The expression of *pax2* reduced significantly ($P < 0.05$) to 50% on silencing at 1 ng concentration, whereas at 10 ng and 100 ng concentrations, the transcript levels were completely reduced ($P < 0.001$) compared with control (Fig. 5A), after 48 h. The *pax2* levels at 10 ng concentration after 24 h reduced to 40%, and these levels were completely knocked down after 48 h compared with control (Fig. 5B). Pax2 protein levels were also significantly reduced at 10 ng concentration after 48 h, and protein levels of Wnt4 were found to be less compared with those of control antibody (Fig. 5C), β -tubulin (Ana Spec Inc., # 55691s), in which no change was observed. Considering these data, primary cultured cells silenced with (Si)*pax2* at 10 ng concentration for 48 h were taken for further analysis of transcript levels of genes chosen for the study. Further, analysis of different transcripts (see below) pertaining to ovary-related genes has strengthened the quality of follicular cell primary culture characteristics.

Change in the expression of various transcripts involved in the ovarian development after silencing *pax2* in primary culture of catfish ovarian follicles, *in vitro*

The transcript levels of *wnt4* (Fig. 5D) and *wnt5* (Fig. 5E) reduced by 50%, significantly ($P < 0.001$) on silencing *pax2* after 24 h, which were further reduced to 80% by 48 h compared with the control in which the control siRNA did not show any significant change (Fig. 5F).

The expression of *star*, *cyp17a1* and *cyp19a1a* reduced by 50%, significantly ($P < 0.001$), whereas the expression of *20 β -hsd* reduced by 30%, significantly ($P < 0.001$), compared with that of the controls (Fig. 6A). *17 β -hsd 1* and *12* significantly ($P < 0.05$) reduced by 20% and 15%, respectively (Fig. 6A). The expression of *foxl2* and *sox9b* significantly ($P < 0.05$) reduced by 30% (Fig. 6B). The expression of *ad4bp/sf-1* reduced by 60%, whereas that of *esr1* reduced significantly ($P < 0.001$) by 30%, and there was no change in *esr2* compared with that in controls (Fig. 6B).

Figure 8 Relative mRNA levels of (A) *star* and steroidogenic enzymes such as *cyp17a1*, *cyp11a1*, *3 β -hsd*, *17 β -hsd1* and *12*, *cyp19a1a* and *20 β -hsd* (B) transcription factors, *foxl2*, *sox9b* and *ad4bp/sf-1* and estrogen receptors, *esr1* and *esr2* in the control and after silencing of *pax2* in catfish. The relative expression was normalized with *18S rRNA* expression, and the values were calculated using comparative ct method. All data were expressed as mean $\pm$ S.E.M. (* $P < 0.05$; ANOVA followed by SNK test). NS, not significant.

Silencing of *pax2* in catfish ovarian follicles, *in vivo* at mRNA and protein levels

The expression levels of *pax2* reduced significantly ($P < 0.005$) by 80% at 1 μ g concentration after 72 h compared with control (Fig. 7A). The expression levels of *pax2*, *wnt4* and *wnt5* did not show any significant change after transfecting with control siRNA (Fig. 7B) compared with those of the respective control sample. The protein levels of Pax2 and Wnt4 also reduced at the same time, whereas there was no change observed for the protein levels of control antibody, β -tubulin (Fig. 7C). Considering this, catfish ovary silenced with (Si)*pax2* at 1 μ g concentration for 72 h was taken for further analysis of transcript levels of several genes chosen for this study. To substantiate our *in vitro* results, we had tested a few other steroidogenic enzyme genes after the silencing of Pax2, *in vivo*.

Change in the expression of various transcripts involved in the ovarian development after silencing *pax2* in catfish, *in vivo*

The expression of *wnt4* and *wnt5* reduced significantly ($P < 0.001$) by 50% and 65%, respectively (Fig. 7A) on silencing *pax2*, compared with the control. The expression of *star* reduced significantly ($P < 0.001$) by 60% compared with that of the control. The expression of *cyp17a1* reduced significantly ($P < 0.001$) by 50%, whereas that of *cyp11a1* increased significantly ($P < 0.001$) to 0.8 fold compared with control (Fig. 8A). *3 β -hsd* did not show any change, whereas 50% significant ($P < 0.05$) reduction was observed in *17 β -hsd 1* and *12* (Fig. 8A). The expression of *cyp19a1a* reduced significantly ($P < 0.05$) by 50%, whereas that of *20 β -hsd* reduced significantly ($P < 0.001$) by 45% compared with control (Fig. 8A). The expression of *foxl2* reduced significantly ($P < 0.05$) by 10% (Fig. 8B). The expression of *sox9b* reduced significantly ($P < 0.05$) by 20% (Fig. 8B) and that of *ad4bp/sf-1* reduced by 65%, significantly ($P < 0.001$), whereas the expression of *esr1* reduced significantly ($P < 0.05$) by 30%, and there was no change in *esr2* compared with that in controls (Fig. 8B).

Change in the levels of steroid hormones, E_2 and $17\alpha,20\beta$ -DP after silencing *pax2* in catfish, *in vivo*

The levels of serum E_2 (Fig. 9A) significantly decreased to 28% ($P < 0.05$) after silencing *pax2*, whereas the levels of $17\alpha,20\beta$ -DP (Fig. 9B) did not show any significant change compared with those of control.

Figure 5

Relative serum levels of (A) estradiol-17 β (E_2) and (B) $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ($17\alpha,20\beta$ -DP) in the control and after silencing of *pax2* in catfish. The relative expression was normalized with *18S rRNA* expression, and the values were calculated using comparative ct method. All data were expressed as mean $\pm$ S.E.M. (* $P < 0.05$; ANOVA followed by SNK test).

Discussion

In this study, the full-length cDNAs of the members of Pax family of transcription factors, *pax1* and *pax2*, and a partial cDNA fragment of *pax9* were obtained from catfish. Considering the predominant expression of *pax2* in ovary in comparison with *pax1* and *pax9*, a detailed study was conducted on the former to analyze its role during ovarian development and recrudescence in catfish. The deduced aa sequence of catfish Pax2 showed its characteristic features of the Pax family with conserved DNA-binding helix-turn-helix motif, Pax and a C-terminal Pax2 domain. Although there are two transcript variants in other teleostean member, *D. rerio*, it is not evident in this species even after repeated RACE analysis and genomic PCR with the primers designed onto two neighboring highly conserved

exons flanking a variable intron. ClustalW alignments showed its homology with the conserved Pax domain of other vertebrates including teleosts.

Expression levels of *pax2* in ovary indicate its plausible role in ovarian development as it plays an important role in thyroid follicle development (Wendl *et al.* 2002), which might have other effects in consequence, as impaired thyroidal status affects gonadal development and recrudescence (Swapna *et al.* 2006, Swapna & Senthilkumaran 2007). In fact, thyroid-stimulating hormone receptor appears to play critical roles in gametogenesis rather than embryogenesis (Goto-Kazeto *et al.* 2009). Higher expression of *pax2* in other tissues like brain and kidney indicates its role as in that of zebrafish, where it has a prominent role for mid-hind brain boundary development (Lun & Brand 1998), and it is required in multiple aspects of pronephros development including tubule and duct epithelial differentiation and cloaca morphogenesis (Majumdar *et al.* 2000). The expression of this transcript in other tissues also supports its role in organ development as being an early expressed embryonic gene. Considering these findings, it is plausible to presume a role for Pax2 in gonadal development. Furthermore, ontogeny analysis indicates its role from the early stage of development of the animals and that it is sex dependent from the critical stage of sexual differentiation and is in favor of females indicating its importance in gonadal differentiation as that of other transcription factors like *foxl2*, *ad4bp/sf-1* and *sox9b* analyzed in catfish (Sudhakumari & Senthilkumaran 2013). The relatively high expression of *pax2* in preparatory and pre-spawning phases of ovarian reproductive cycle of catfish indicates its prominent role in recrudescence. Although the relative levels of *pax2* in testis are low compared with those in ovary during reproductive cycle, its presence may contribute to testicular development. However, its putative role is not yet known. Induction of *pax2* levels using hCG indicates its role in pre-spawning/spawning and its regulation by gonadotropins. Localization of mRNA through ISH in ovary showed its expression in peri-nucleolar, pre-vitellogenic and vitellogenic oocytes, and follicular layer of vitellogenic and post-vitellogenic oocytes. Similar pattern was evident during immunoreactivity with heterologous antibody in oocytes, and also a clear protein band from ovarian protein lysate could be observed. Expression levels in different stages of follicles seem to be of high quantities earlier in developmental stages. By and large, these results creditably support the prime significance of Pax2 in ovarian development and recrudescence.

To analyze its function in ovarian development, *pax2* transient silencing experiments were performed in catfish primary culture of ovarian follicles, *in vitro*, and also through ovary-targeted injection of siRNA into the catfish, *in vivo*. Although different cell types from follicular primary culture were not characterized, steady expression of *pax2* and measurement of different ovary-related transcripts confirm the stability. To alleviate the off-target effects, we used custom-synthesized catfish-specific esiRNA, which is an endoribonuclease-prepared heterogeneous mixture of siRNA pools with multiple triggers that target the same mRNA sequence. In addition, control esiRNA was used to validate our findings. After silencing of *pax2* mRNA and protein levels, the expression patterns of Wnt signaling molecules, various transcripts encoding steroidogenic enzymes and transcription factors, which are known to be essential for ovarian development in catfish were analyzed. There is a 50% reduction in the expression pattern of most of the transcripts analyzed both *in vitro* and *in vivo* indicating that *pax2* affects their expression through direct or indirect action. Upon silencing Pax2, down regulation of the expression of signaling molecules, *wnt4* and *wnt5*, indicates a direct interaction of Pax2 in catfish as proved in the kidney of mammals (Torban *et al.* 2006, Tamimi *et al.* 2008). There is a reduction in the gene encoding Star, a transport protein for cholesterol within the mitochondria, which is a rate-limiting step in steroidogenesis (Sreenivasulu *et al.* 2009). This change might have been due to Ad4bp/sf-1, which plays an important role in the regulation of many steroidogenic enzymes, especially cytochrome P450 steroid hydroxylases/aromatase (Yoshiura *et al.* 2003, Senthilkumaran *et al.* 2004) and steroidogenic acute regulatory protein, which might have reduced the transcript levels of *cyp17a1* and *star* (Raghuveer *et al.* 2011). The decreased expression of transcription factor, Foxl2, a member of the forkhead/HNF-3-related family, which is involved in ovarian differentiation and many other developmental processes (Sridevi & Senthilkumaran 2011) might have reduced the expression of *ad4bp/sf-1*, which eventually downregulated *cyp19a1a* transcription as they transcriptionally interact together during ovarian recrudescence of teleosts (Yoshiura *et al.* 2003, Yamaguchi *et al.* 2007, Wang *et al.* 2007, Rasheeda *et al.* 2010). Decreased expression of another transcription factor, Sox9b that is critical for ovarian differentiation in catfish (Raghuveer & Senthilkumaran 2010) might also be attributed to the low expression of *ad4bp/sf-1*. Interestingly, there is no change in the

expression of *3 β -hsd*, whereas *cyp11a1* was upregulated after Pax2 silencing in catfish, *in vivo*. Although the reason for such differential response is not clear, it reiterates that the siRNA of *pax2* treatment resulted in a varied response. Interestingly, both the steroidogenic enzyme genes play an essential role in the gonadal development and recrudescence in catfish (Raghuveer & Senthilkumaran 2012, Rajakumar & Senthilkumaran 2014b). There is a decrease in transcript levels of steroidogenic enzymes, 17 β -hsd1 and 12, which may perhaps modulate gametogenesis and gamete maturation in catfish (Rajakumar & Senthilkumaran 2014c). There is a reduction in the expression pattern of *20 β -hsd*, the enzyme responsible for oocyte maturation in teleosts, along with *cyp19a1a* and *cyp17a1* (Senthilkumaran *et al.* 2004, Sreenivasulu & Senthilkumaran 2009a,b), which were effected through *ad4bp/sf-1*, indicating the role of *pax2* in oocyte growth and maturation as a consequence of events. Nevertheless, it remains to be seen whether *pax2* also regulates CREB, which controls the oocyte growth and maturation in teleosts (Senthilkumaran *et al.* 2015). There is a reduction in the expression of *esr1*, in accordance with the steroidogenic enzyme genes, which might indicate probable intermediate actions in imparting its effects on ovarian development, where *esrs* serve as ligand-dependent transcription factors for estrogen secretion (Parker *et al.* 1991, Filby & Tyler 2005). There is no change in *esr2* that might have compensating action for *esr1* changes. Marginally significant reduction in the levels of E_2 after silencing *pax2* might validate altered transcript levels of steroidogenic enzymes responsible for E_2 production. Reduction in the transcript levels of *20 β -hsd*, the enzyme responsible for oocyte maturation did not alter 17 α ,20 β -DP levels indicating that the transient silencing at the recrudescence stage (mid-preparatory phase) of fish might have less immediate effects at functional level.

Taken together, it is possible to suggest that the modulation of transcripts involved in ovarian steroidogenesis might have been due to Pax2 regulation either on direct binding with proximal promoters of steroidogenic enzyme genes, as there is the presence of Pax2-binding motif in steroidogenic enzyme genes and also indirectly through Pax2-mediated *wnt4* downregulation as that of zebrafish and mammals in which *wnt4* is known as a probable regulator of steroidogenesis (Kocer *et al.* 2009, Sreenivasan *et al.* 2014). It is possible that in teleosts too, Pax2 might operate its signaling through *wnt4/5* as reported in mammals (Zhou 2012). In fact, our extended work on this line identified functional

Pax2-binding site in *wnt5* promoter motif with a synergistic expression pattern (Prathibha Y & Senthilkumaran B, unpublished observations). The effects on steroidogenesis are perhaps partly mediated through Wnt signaling in a Pax2-dependent manner accrediting the importance of *pax2* in ovarian development and recrudescence of catfish. Although these findings provide introductory evidence on the interaction of Pax2-*wnt4/5* in terms of ovarian steroidogenesis and development, this study for the first time provides a basis for such interaction in a lower vertebrate using transcript level analysis. These results may not implicate the absence of regulation of steroidogenesis by Pax2 in testis as there is a commonality of the endocrine processes in both sexes, which needs to be explored in detail.

Conclusion

This study demonstrates that *pax2* plays an important role in ovarian development and recrudescence of catfish by regulating steroidogenesis either directly or indirectly through Wnt signaling pathway as evident from transient gene-silencing experiments in catfish, *in vivo* and ovarian follicle primary culture, *in vitro*.

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.

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