Expression of *wnt4/5* during reproductive cycle of catfish and *wnt5* promoter analysis

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Abstract

Signaling molecules, Wnt4 and Wnt5, are essential for ovarian growth during developmental stages in mammals. Although these molecules were identified in several teleosts, their precise expression and role in reproductive processes have not yet been explored in any lower vertebrates. In view of this, using catfish, *Clarias batrachus* as an animal model, cloning and expression analysis of *wnt4* and *wnt5* were analyzed in different tissues, at various developmental stages, during ovarian reproductive cycle and after gonadotropin induction. These studies indicate a plausible influence of Wnts in ovarian development and recrudescence. Transcript and protein localization revealed their presence in peri-nucleolar, pre-vitellogenic, vitellogenic and follicular layer of post-vitellogenic oocytes. Synchronous expression of *pax2* and *wnt5* during the ovarian development and recrudescence of catfish led us to analyze the importance of putative binding element of Pax2 in the 5′-promoter motif of *wnt5*. Promoter activity of *wnt5* was analyzed by luciferase assays after transfecting progressive deletion constructs in pGL3 basic vector into the mammalian cell lines (HEK 293 and CHO). The constructs having putative Pax2 motif showed high promoter activity compared with controls. Likewise, the constructs with site-directed mutagenesis showed increased activity after supplementing recombinant Pax2 indicating the prominence of this motif in *wnt5* promoter, *in vitro*. Electrophoretic gel mobility shift, supershift and chromatin immunoprecipitation assays confirmed the binding of Pax2 to its corresponding cis-acting element in the upstream of *wnt5*. This study is the first of its kind to report the critical transcriptional interaction of Pax2 on *wnt5* vis-à-vis ovarian development in teleosts.

Key Words

- ovarian follicles
- Wnt signaling
- paired box
- teleost
- putative binding motif

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Introduction

Ovarian development is regulated by various signaling pathways along with other growth factors. Wnt/Frizzled pathways play an important role in ovarian embryogenesis, folliculogenesis and possibly ovulation and luteinization in mammals (Richards *et al.* 2002, Boyer *et al.* 2010*a*). The precise roles of these signaling molecules

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in high-fecundity species like fishes are least understood. Although 14 members of these secreted signaling molecules of Wnt family were identified in teleosts (Yokoi *et al.* 2003), their functions were not specified in lower vertebrates, at least through expression analysis. Of these, Wnt4 and Wnt5 seem essential for gonadal differentiation and embryogenesis, respectively, in a few species (Kilian *et al.* 2003, Eggers *et al.* 2014), but their role during the annual reproductive cycle has not yet been explored.

Wnt4 is one of the essential factors for embryogenesis and sex determination/differentiation events of all vertebrates (Eggers *et al.* 2014). In mammals, the function of *WNT4* is well studied in female reproductive system, where it is known to be the signal molecule for male–female phenotype conversion and plays an important role in steroidogenesis by the suppressing Leydig cell development in mice (Kocer *et al.* 2009). In humans, it is required for oocyte selection, follicle formation and maturation (Boyer *et al.* 2010*b*, Jaaskelainen *et al.* 2010, Prunskaite-Hyyryläinen *et al.* 2014). The precise functions of this gene in teleosts are not well explored in reproduction although it is identified in several teleosts such as zebrafish (Ungar *et al.* 1995), rainbow trout (Nicol *et al.* 2012), medaka (Yokoi *et al.* 2003), half-smooth tongue sole (Hu *et al.* 2014), black porgy (Wu & Chang 2009) and hermaphrodite and orange-spotted grouper (Chen *et al.* 2015). Two paralogs of this gene were reported in zebrafish, medaka and half-smooth tongue sole with variant functions during development. *wnt4a* along with other *wnt* forms is known to regulate midline convergence of organ primordia during zebrafish development (Matsui *et al.* 2005), whereas *wnt4b* is required for floor plate development in zebrafish (Liu *et al.* 2000) and medaka (Inohaya *et al.* 2010). *wnt4a* is reported to be essential for embryogenesis and gonadal differentiation in zebrafish (Ungar *et al.* 1995, Sreenivasan *et al.* 2014). The expression pattern in rainbow trout showed that it is required for both male and female development (Nicol *et al.* 2012). In black porgy, it seems to be essential for ovarian growth (Wu & Chang 2009).

The other signaling molecule, Wnt5 plays various roles in vertebrate development. Two paralogs of this gene (Wnt5a and Wnt5b) are reported in mammals and a few teleosts with varied physiological functions and operate different signaling pathways. In mammals, it is required for the development of pancreas (Kim *et al.* 2005) and kidney (Huang *et al.* 2014). In zebrafish embryogenesis, it plays a critical role in conversion and extension movements of the embryo (Kilian *et al.* 2003) along with other signaling molecules (Liu *et al.* 2009). It is also required for tail formation (Rauch *et al.* 1997) and regulates cell elongation and convergent extension movements in the posterior regions of the gastrula. The essential role of this molecule with respect to reproduction is not reported well in teleosts; however, it coordinates with Wnt4 for the initiation of meiosis in mammals for ovarian follicular growth (Naillat *et al.* 2010).

Here, we made an attempt to understand the role of these signaling molecules, Wnt4 and Wnt5, during the ovarian development and recrudescence of catfish, *Clarias batrachus*, an annual breeder, through expression analysis under different physiological status. For this, full-length cDNAs were cloned and the transcript levels in various tissues and developmental stages were analyzed. To understand their role in ovarian development, ontogeny, phase-wise expression and human chorionic gonadotropin (hCG) induction studies were performed. The localization of their transcripts through *in situ* hybridization (ISH) and Wnt4 protein localization through immunohistochemistry (IHC) were done. These findings provide a basis to examine the possible regulation of Wnt molecules at the promoter level for mRNA and protein synthesis. Considering the lack of information at the promoter level for any signaling molecules in teleosts, *wnt5* seems to be an ideal candidate for the analysis. Hence, the present report also analyzed the transcriptional interactions of Pax2 with the promoter of *wnt5* in catfish. Even though *wnt5* is a member of early expressed genes, its interaction with other molecules or transcription factors in teleosts during the developmental processes have not been evaluated. In this context, it is worthwhile to study the possible involvement of other transcription factors in *wnt5* regulation at the promoter level, which might provide valuable insight into the regulatory roles of specific upstream factors in eliciting their effects for ovarian growth and maturation. As the ovarian development in teleosts is a complex process, the information on the transcription factors regulating this mechanism is limited to very few studies. *In silico* and functional studies on various genes in teleost ovary revealed interactions with different transcription factor-binding sites (Hu *et al.* 2001, Parker *et al.* 2002, Yoshiura *et al.* 2003, Wang *et al.* 2007, Sreenivasulu *et al.* 2012, Senthilkumaran *et al.* 2015). Hence, to figure out other interactions involved in these processes, this study was conducted using catfish, *C. batrachus*, to provide a basic understanding on the implication of signaling molecule with the regulation by transcription factors

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during ovarian development, maturation and seasonal reproductive cycle in teleosts.

Materials and methods

Animals and sampling

Fingerlings and adults of catfish, *C. batrachus*, used in the present work were obtained by *in vitro* fertilization established in our laboratory (Rajakumar *et al.* 2012) with the brood stock purchased from the markets of local fishermen of river Ganges. Fingerlings rearing till adulthood and feeding was explained earlier (Rajakumar *et al.* 2012). Tissue distribution study was performed during the preparatory phase of reproductive cycle using ovary, brain, liver, kidney, gills, intestine, muscle, heart and spleen of female and testis of male adult catfishes (n=10). For ontogeny analysis, hatched larvae (n=15) was taken and considered as 0 days post hatch (dph), whereas mesonephric gonadal complex from 5, 10, 30 and 40 dph fingerlings (n=15) were dissected out carefully by removing digestive tract and muscles. Developing gonads (n=15) from the sexual differentiation stage of 50 dph to the adult (50, 100, 150, 200, 250 and 365 dph) from males and females were isolated separately. Ovaries were collected from catfish preparatory (February–April), pre-spawning (May–June), spawning (July–August) and post-spawning/resting phases (September–January) as per the seasonal reproductive cycle pattern characterized in our laboratory. Animal maintenance, experimentation and killing were done following the general institutional animal ethical committee guidelines, University of Hyderabad.

Cloning of *wnt4* and *wnt5* from catfish ovary

Total RNA isolated using TRI reagent (Sigma) from ovary of catfish was used for cDNA synthesis with SuperScript III according to the manufacturer's instructions (Invitrogen), followed by DNase I treatment to exclude DNA contamination. Partial cDNA fragments of both *wnt4* and *wnt5* were obtained with a set of respective degenerate primers (*wnt4* Deg fw & *wnt4* Deg rv; *wnt5* Deg fw & *wnt5* Deg rv; Supplementary Table 1, see section on supplementary data given at the end of this article) designed by aligning the available nucleotide sequences from NCBI GenBank database through MegAlign software. The full-length cloning was done by following protocols of RACE (Clontech). List of primers used in RACE (*wnt4* 5P, *wnt4* 5N, *wnt4* 3P and *wnt4* 3N; *wnt5* 5P, *wnt5* 5N, *wnt5* 3P and *wnt5* 3N) have been provided in Supplementary Table 1. The analysis of sequence homology of the cloned cDNA sequences to *wnt4* and *wnt5* of other vertebrates were carried out using NCBI-BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the assembled sequences were translated using EditSeq of Lasergene 7.1.0 (DNASTAR). The probable existence of other paralogs of these genes was tested by designing primers with the alignment of neighboring exons with a flanking variable intron in the conserved Wnt domain followed by their genomic DNA PCR. Primers were designed by aligning Wnt4 and Wnt5 sequences of species with the available genomic information (human and mouse), and their sequence homology with other teleosts as basis. Genomic DNA was isolated from catfish ovary, and the reactions were performed under standard PCR conditions. A single band was observed for both genes indicating the absence of other paralogs. The analysis of homology of the deduced amino acid sequence of catfish *wnt4* and *wnt5* with that of other teleosts was carried out using ClustalW multiple alignment tool using default parameters.

Phylogenetic analysis

Multiple alignment of deduced amino acid sequences followed by their phylogenetic analysis was performed for Wnt4 and Wnt5 with their counterparts from other teleosts and mammals obtained from GenBank. Multiple alignment was performed with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). 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 PCR

With 1 µg of total RNA, extracted using TRI reagent (Sigma) as per the manufacturer's instructions, reverse transcription was performed with verso reverse transcriptase (Thermo Scientific) with random hexamers. For all quantitative PCR (qPCR) experiments, the specific primers (*wnt4* RT fw and *wnt4* RT rv; *wnt5* RT fw and *wnt4* RT rv; Supplementary Table 1) used were designed for the amplicon length of ~150–250 bp. Power SYBR Green PCR Master Mix (Applied Biosystems) was used for all the reactions performed in triplicates for each biological

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replicate in an ABI Prism 7500 fast thermal cycler (Applied Biosystems) according to the manufacturer's universal thermal cycling conditions. As per the validation performed based on Radonić *et al.* (2004), *18S rRNA* was taken for normalization, which was found to be more efficient and constitutive from our preliminary experiments of qPCR with several reference genes such as *18S rRNA* (KM018296.1), β-*actin* (EU527190.2) and *gapdh* (KC414932.1) cloned from catfish. The specificity and efficiency of all the primers were checked using semi-quantitative PCR before proceeding to qPCR. After performing melting-curve analysis to check the specificity of PCR amplification, cycle threshold (Ct) values obtained from the exponential phase of PCR were used for generating ΔCt value where *18S rRNA* is taken for the normalization against the expression of target gene. The gene expression was evaluated by $2^{-\Delta Ct}$ method.

Induction by hCG

During the mid-preparatory phase (March), laboratory-acclimated female adult catfishes (n=6) weighing about 170–200 g were intraperitoneally injected a single dose (1000 IU/kg body weight) of hCG (Pubergen, Uni-Sankyo Pvt. Ltd., Hyderabad, India) or saline. Fishes were killed in different intervals (0, 6, 12, 18 and 24 h) for control and treated separately. Total RNA isolation, reverse transcription, qPCR analysis and its evaluation were performed as described earlier.

ISH and IHC

Digoxigenin labeling method was used for localization of transcripts of *wnt4* and *wnt5* through ISH in the ovary of adult catfish taken during pre-spawning phase. ISH protocol was followed as per the method described in Rajakumar and Senthilkumaran (2014). Localization of Wnt4 on ovarian sections and their photographing were done as explained earlier in Rajakumar and Senthilkumaran (2014). The polyclonal Wnt4 antibody (LifeSpan Biosciences Inc, Seattle, WA, USA; LS-C109642/61282) used for IHC was raised against the conserved Wnt domain of human WNT4 that showed 80% homology with the conserved region of catfish Wnt4.

Isolation of 5′ upstream region of *wnt5* and *in silico* analysis

The genome libraries prepared from the genomic DNA of adult catfish testis (Rajakumar & Senthilkumaran 2016) as per the manufacturer's protocol of Universal Genome Walker kit (Clontech) were used for the isolation of 5′ upstream region of *wnt5*. Four genomic libraries used were obtained after digesting aliquots of genomic DNA (25 µg) overnight with *Eco*RV, *Pvu*II, *Dra*I or *Stu*I and were ligated separately to the Genome Walker adaptor. Using adaptor primers and gene-specific primers designed from open reading frame (ORF) of *wnt5* (Supplementary Table 1), primary (AP1, 5PW) and secondary (AP2, 5NW) PCR amplifications were carried out with the prepared libraries individually. Standard cycling conditions were performed at 94°C 30 s, 72°C 3 min, 5 cycles, 94°C 30 s, 68°C 30 s and 72°C 3 min for 30 cycles, whereas secondary PCR amplicons were cloned into pGEM-T Easy vector (Promega) and sequenced bi-directionally using dye terminator cycle sequencing method in an ABI 3730 DNA analyzer (Applied Biosystems). Promoter prediction was carried out using the neural network promoter prediction and McPromoter programs. Putative transcription factor-binding sites were predicted using GP Miner and MatInspector matrix family library version 9.0 of MatInspector professional 8.06 (Genomatix Software Suite, München, Germany). The parameters were set to predict general core promoter elements for vertebrates with maximum score of 1.0 and minimum of 0.75. The program output generated a table of matrices with several predicted transcription factor-binding sites, but only those with high core similarity, together with possible importance in reproductive/steroidogenic processes, were chosen for further functional analysis. After two rounds of PCR amplifications, using the libraries constructed, the product (~659 bp) was cloned, which corresponds to the 5′ upstream region of *wnt5*, and the same was submitted to GenBank (KX529113). *In silico* promoter motif analysis revealed the presence of binding motifs of Pax2, Sox9b, FOXO1, GATA1/6 and TATA box (Supplementary Fig. 2A). The putative transcription start site (+1) of *wnt5* was predicted based on the 5′ RACE sequence data. The schematically presented (Supplementary Fig. 2B) progressive PCR deletion constructs were prepared with respect to the binding sites and were confirmed with gel electrophoresis (Supplementary Fig. 2C) followed by sequencing.

Plasmid constructs and site-directed mutagenesis (SDM)

Restriction site flanked gene-specific primers (DC1F, DC2F, DC3F and DCR) designed from the *wnt5* upstream region (Supplementary Table 1) were used for the amplification of progressive deletion constructs, 1, 2 and 3 (DC1, DC2 and DC3) from 5′ upstream region of *wnt5*. KpnI and NheI

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site-restricted primers were used for double digestion, and their subsequent cloning into KpnI and NheI sites of pGL3-basic vector (Promega). The identity of each construct and the artifacts in the sequence were verified by plasmid DNA sequencing. SDM (Q5 NEB) was carried out to mutate the specific Pax2 TF-binding site. Luciferase plasmid constructs bearing mutation in Pax2-binding motif were constructed by PCR-based mutagenesis using primers (SDMF and SDMR; Supplementary Table 1) designed to contain mutated bases by substitution (NEB base changer). ORF of catfish Pax2 was cloned into pcDNA3.1 using pcPax2 Fr and pcPax2 Rv primers (Supplementary Table 1) and was used for overexpression studies by co-transfection along with deletion constructs, whereas pcDNA with non-coding Pax2 ORF reversed was used as a negative control.

Cell culture

The cell lines, Chinese hamster ovary (CHO) and human embryonic kidney (HEK 293), were procured from National Centre for Cell Sciences (Pune, India). They were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco-BRL, Invitrogen), 2mM L-glutamine and antibiotic and antimycotic solution (Gibco-BRL) at 37°C and 5% CO_2.

Transient transfection and luciferase reporter assay

HEK293 and CHO cells cultured as mentioned previously were transfected plasmid constructs (500ng) and pcDNA-Pax2 plasmids (100ng) using Lipofectamine 2000 reagent (Invitrogen) as per the manufacturer's protocol. pRL-TK plasmid (10ng) expressing *Renilla* luciferase under herpes simplex virus thymidine kinase promoter was used as an internal control. Promoter activity analysis was performed as per the method described earlier (Sreenivasulu *et al.* 2012). After transfection (~36h), cells were washed once with PBS and lysed with 120µL (per well) of passive lysis buffer (Promega) for 20min at 37°C in a rocker. The supernatant collected after cell lysis from plates by vortex and brief spun at 4°C (12,000***g***, 30s) was used to check promoter activity. Dual-luciferase reporter assay system (Promega) was used to quantify the reporter gene expression by GloMax 20/20 Single Tube Luminometer (Promega) as per the manufacturer's protocol. Average of firefly luciferase activity expressed as the ratio to the *Renilla* luciferase activity was presented as results. All experiments were done with three independent samples in triplicates.

Electrophoretic mobility shift assay (EMSA)

Catfish ovarian nuclear extract preparation and EMSA were performed by following the method of Smith and Delbary-Gossart (2001) and Sreenivasulu *et al.* (2012) with minor modifications. Specific oligonucleotides corresponding to the Pax2 site were designed with spanning Pax2-binding motif (EMSA N) and modification by substitution was done for mutant probe (EMSA M; Supplementary Table 1) and were synthesized and annealed into double strands. Radiolabeled probes generated by incubating 250ng of annealed oligonucleotides with 20µCi [γ-^{32}P] dATP in the presence of T4 Polynucleotide Kinase (Fermentas) for 30min at 37°C were used after subsequent separation from free nucleotides using G-25 column purification (GE-Healthcare, Amersham Biosciences). Catfish ovarian nuclear extract (25µg) was incubated with ~20fmol of [γ-^{32}P] end-labeled wild or mutated Pax2 oligonucleotides (EMSA N and EMSA M; Supplementary Table 1) in the presence of 0.5µg of poly(dI–dC) in binding buffer (20mM HEPES, pH 7.9, 0.5mM EDTA, 0.4mM DTT and 7.5% glycerol) for 30min at 37°C. A 6% native polyacrylamide gel was used for separation of DNA–protein complex in 0.5× Tris-borate EDTA as running buffer. For cold competition, the nuclear extract was incubated with different concentrations of unlabeled/cold (ds) oligos in binding reaction before the addition of radiolabeled probe. Pax2 antibody (Life Span Biosciences Inc. LS-C102856) was used for supershift binding reaction by subsequent incubation for 30min at RT. After electrophoresis, gels were exposed to a phosphor imager cassette and scanned using Typhoon Trio$^+$ variable mode imager (GE Healthcare, Amersham Biosciences).

Chromatin immunoprecipitation assay (ChIP)

ChIP was performed as per the protocol of Turner *et al.* (2006) with a few modifications using adult catfish ovary. In brief, formaldehyde crosslinking was done with 25mg of homogenized catfish ovarian tissue by supplementing with protease inhibitors (1mM PMSF, 1µg/mL aprotinin, 1µg/mL pepstatin and 1µg/mL leupeptin). The cell pellet obtained after centrifuging for 15min at 450***g***, was suspended in lysis buffer (150mM NaCl, 25mM Tris–HCl, pH 7.5, 5mM EDTA, pH 8.0, 1% Triton-X-100, 0.1% SDS, 0.5% sodium deoxycholate with protease inhibitors) and sonicated on ice with 10×15-s bursts with a 30-s pause. The sonicated samples were diluted in lysis buffer and were used for preclearing using pre-immune serum and protein-A agarose beads for 1h at 4°C. Pre-cleared extracts were then incubated with

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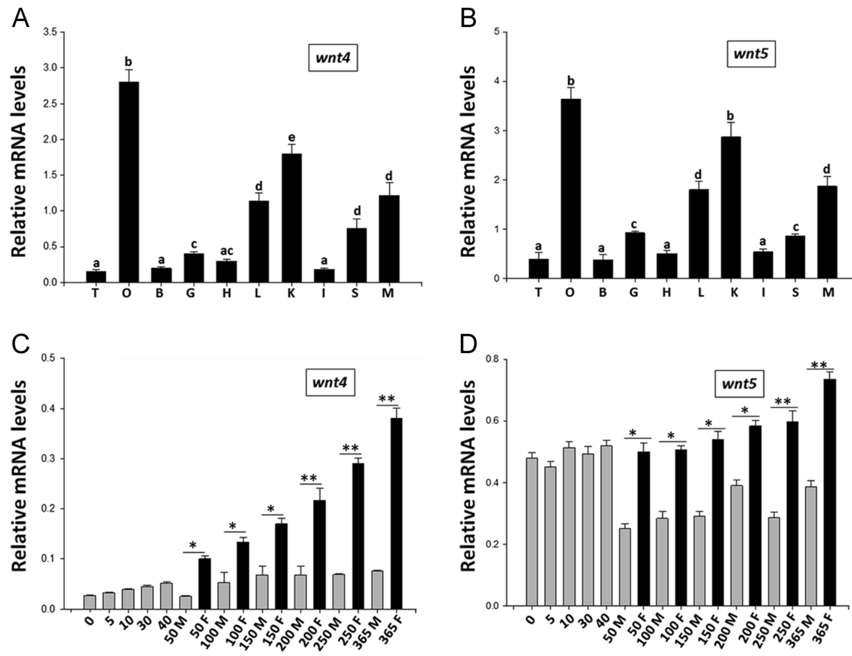

Figure 1
Relative mRNA levels of (A, C) *wnt4* and (B, D) *wnt5* in various tissues and during different stages of gonadal development of catfish between 0 and 365 days after hatch at diverse time points. The relative expression was normalized with *18S rRNA*, and the values were calculated using comparative Ct method. All data were expressed as mean ± S.E.M. 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 *wnt4/5* mRNA levels when compared with the corresponding opposite sex of the same age group ($P < 0.05$; $P < 0.01$; ANOVA followed by SNK test). B, Brain; F, Female; G, Gill; H, Heart; I, Intestine; K, Kidney; L, Liver; M, Male; M, Muscle; O, Ovary; S, Spleen; T, Testes.

either anti-IgG or anti-Pax2 or without the addition of any antibody (no antibody control) overnight at 4°C with end-on-end rotation. Washes were carried out according to the protocol given in Turner *et al.* (2006). DNA was isolated from the Pax2 pull-down chromatin and used for PCR amplification. The specific primers (W5 ip Fr and W5 ip Rv; Supplementary Table 1) used for ChIP assay for detection of Pax2-binding sites were designed from *wnt5* promoter with spanning putative Pax2-binding motif. PCR conditions were as follows: 94°C for 4 min, 35 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 20 s and the final step at 72°C for 2 min. qRT-PCR of the immunoprecipitated samples were performed, and the values were plotted as percent input.

Statistical analysis

All data were presented as mean of different samples with standard error of the mean (mean ± S.E.M.). All data were compared by one-way ANOVA by ranks followed by SNK *post hoc* test. All statistical analyses were performed using SigmaPlot 11.0 software (Systat Software Inc., Chicago,

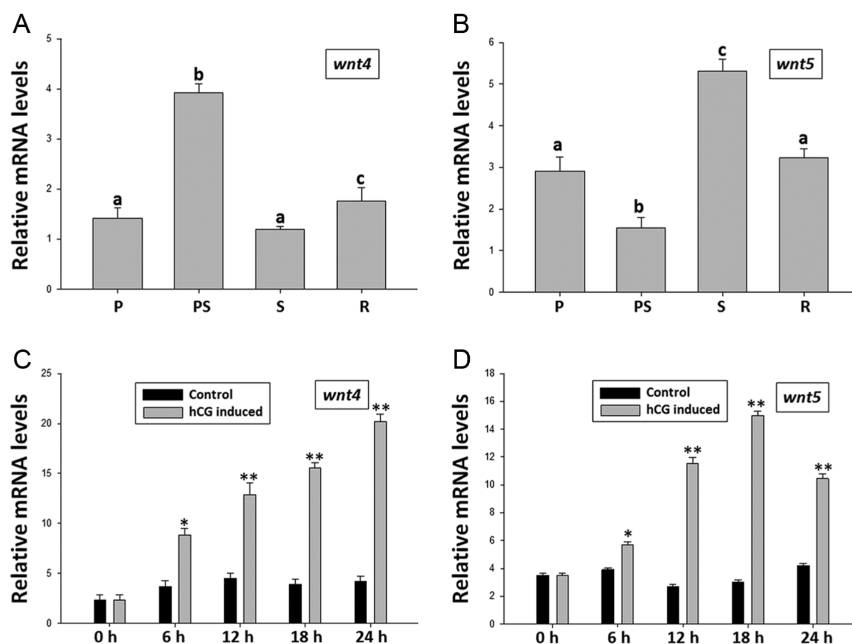

Figure 2
Relative mRNA levels of *wnt4* (A, C) and *wnt5* (B, D) during various phases of the ovarian reproductive cycle and at different intervals after hCG induction. The relative expression was normalized with *18S rRNA* and the values were calculated using comparative Ct method. All data were expressed as mean ± S.E.M. 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 *wnt4/5* mRNA levels compared with the 0 h and respective control ($P < 0.05$; $P < 0.01$, ANOVA followed by SNK test). P, Preparatory; PS, Pre-spawning; R, Post-spawning/Resting Phase; S, Spawning.

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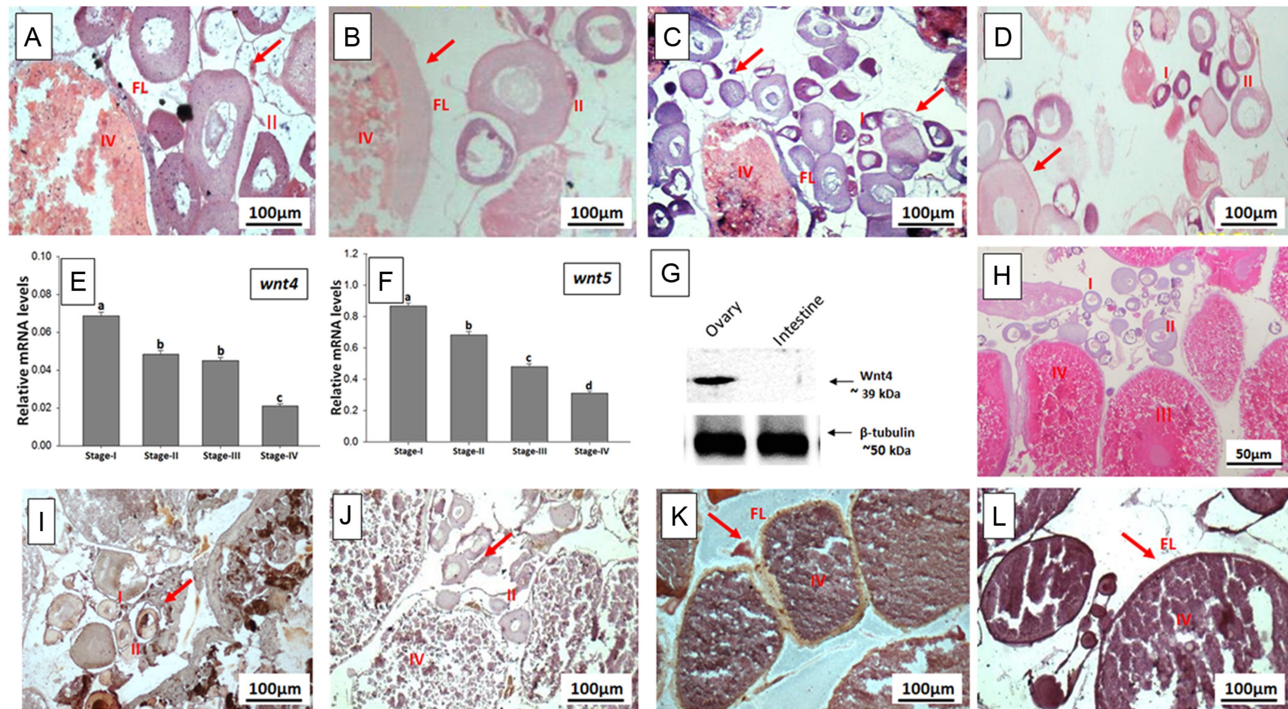

Figure 3
Localization of mRNAs of *wnt4* (A, B) and *wnt5* (C, D) with anti-sense and sense probes in the adult catfish ovary by ISH. Relative mRNA levels of *wnt4* (E) and *wnt5* (F) in the ovary of catfish at various developmental stages of oocytes. The relative expression was normalized with *18S rRNA* and the values were calculated using comparative Ct method. All data were expressed as mean ± S.E.M. Means with different letters differ significantly ($P<0.05$; ANOVA followed by SNK test). (I) Perinucleolar, (II) Pre-vitellogenic, (III) Vitellogenic and (IV) Post-vitellogenic oocytes. FL, Follicular layer. (G) Western blot analysis demonstrating the antibody specificity of Wnt4. (H) Hematoxylin and eosin staining of ovary showing stages of oocytes. (I, K) Localization of Wnt4 in the adult catfish ovary by IHC with anti-Wnt4. (J, L) Pre-adsorbed antibody of Wnt4 with excess Wnt4 peptide elicited no immunoreactivity. Arrows indicate ovary sections showing the presence or absence of transcript localization/immunoreactivity. A full colour version of this figure available at http://dx.doi.org/10.1530/JOE-16-0104.

IL, USA). A probability of $P<0.05$ was considered statistically significant.

Results

Cloning and phylogenetic analysis of *wnt4* and *wnt5* from catfish ovary

ORFs of ~1 kb and ~1.1 kb were obtained for *wnt4* and *wnt5*, respectively, from 5′ and 3′ UTRs and their full-length cDNA sequences obtained were submitted to GenBank (*wnt4* (KX499465), *wnt5* (KX529112)). The deduced amino acid sequence possesses characteristic Wnt domain and 24 cysteine residues along with the signal peptide. To isolate the paralogs of these genes, was performed genomic DNA PCR was performed with the primers designed from highly conserved exons with variable intron which yielded no paralogs. Phylogenetic analyses indicate high homology of Wnt4 with zebrafish and other teleost Wnt4a (Supplementary Fig. 1A), whereas Wnt5 has similarity with Wnt5a (Supplementary Fig. 1B). Lack of isolation of paralogs led us to retain the names as Wnt4 and Wnt5.

Expression of *wnt4* and *wnt5* in different tissues, during ontogeny and different phases of reproductive cycle and after *in vivo* hCG induction

Expression analysis of *wnt4* (Fig. 1A) and *wnt5* (Fig. 1B) in different tissues of adult catfish in the preparatory phase showed that it is predominantly expressed in ovary followed by kidney, liver, muscle and spleen. The expression was minimal in other tissues analyzed. The ontogenic expression at various developmental stages from 0 dph to adult showed dimorphic expression in favor of females from 50 dph, the critical period of sex differentiation of catfish, and it is maintained till adulthood (Fig. 1C and D). qPCR analysis showed high levels of *wnt4* in pre-spawning phase (Fig. 2A) and *wnt5* in spawning phase (Fig. 2B) of catfish ovarian cycle. The *in vivo* induction of hCG at various intervals showed a

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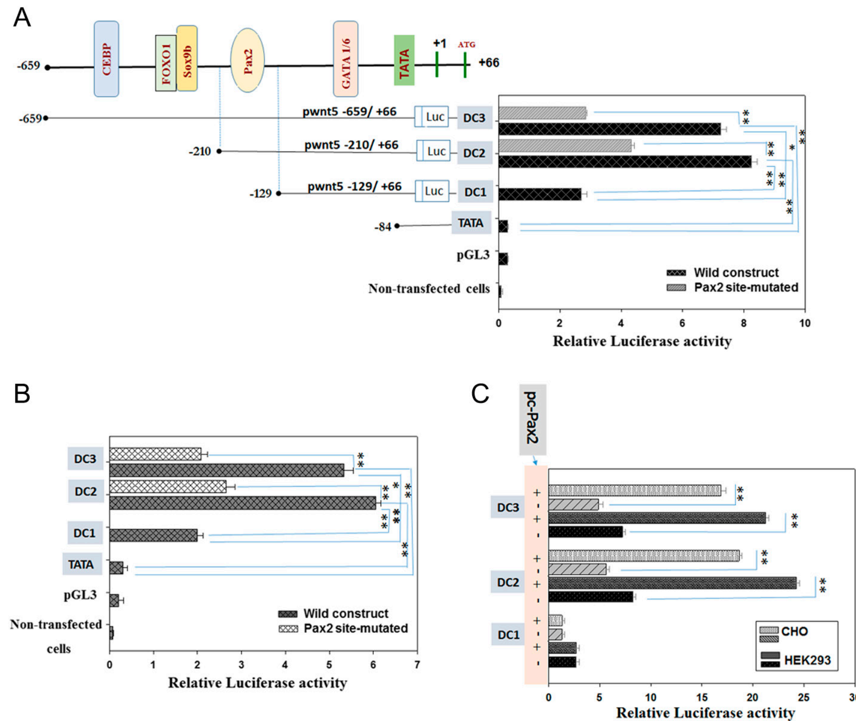

Figure 4
Functional activity of wild and mutated Pax2-binding site in (A) HEK293 and (B) CHO cells. Progressive *wnt5* promoter wild (DC3, DC2 and DC1) and mutated deletion constructs in pGL3 basic plasmid (500 ng each) were transfected into the cell lines separately. (C) Functional activity of wild Pax2-binding sites with Pax2 overexpression in HEK293 and CHO cells. pGL3 basic vector with *wnt5* promoter wild deletion constructs, DC3, DC2 and DC1 (500 ng each) co-transfected with Pax2 expression vector (pc-Pax2Fr, 100 ng each; indicated as +) into the cell lines. – indicates pcDNA-Pax2Rv with non-coding ORF plasmid co-transfection along with deletion constructs (DC3, DC2 and DC1). Luciferase activity was presented as fold change relative to the activities measured for *Renilla* luciferase. The data represent mean ± S.E.M. from three independent experiments performed in triplicate. */** indicates means with significantly different luciferase activity ($P < 0.05$; $P < 0.01$; ANOVA followed by SNK test). A full colour version of this figure available at http://dx.doi.org/10.1530/JOE-16-0104.

significant ($P < 0.05$; $P < 0.01$) increased expression in a time-dependent manner in ovary up to 24 h compared with 0 h and respective controls for *wnt4* (Fig. 2C), whereas for *wnt5*, the transcript levels reduced by 24 h (Fig. 2D).

Localization and quantitation of mRNAs of *wnt4* and *wnt5* and the localization of Wnt4 proteins

Localization of *wnt4* and *wnt5* transcripts (Fig. 3A and C) indicated their presence in peri-nucleolar, pre-vitellogenic and vitellogenic oocytes and follicular layer of post-vitellogenic oocytes, which is verified with no signal in sense probe (Fig. 3B and D). It is also interesting to note that the transcript localization gradually reaches follicular layer in accordance with the growth of oocytes. The qPCR analysis for the same indicated their higher expression in pre-vitellogenic oocytes followed by vitellogenic oocytes (Fig. 3E and F). The hematoxylin and eosin section of ovary was also provided to represent the stages of oocytes (Fig. 3H). The heterologous Wnt4 antibody used for protein localization showed clear protein band of ~39 kDa by western blot in ovary (Fig. 3G), which can be confirmed with the absence in intestine protein extract used as negative control. β-Tubulin was shown for equal loading of samples. The immunoreactivity observed for Wnt4 protein (Fig. 3I and K) confirmed its protein localization as that of its transcripts, whereas pre-adsorbed antibody with excess peptide of Wnt4 gave no signal (Fig. 3J and L).

Identification of functional Pax2-binding motifs in the upstream region of *wnt5*

The construct with putative Pax2-binding motif showed a significantly high ($P < 0.05$) promoter activity in both the cell lines (Fig. 4A and B). The construct with TATA box showed only minimal activity. The presence of other sites, Sox9b, FOXO1, CEBP and GATA1/6, may contribute additive effects in the promoter activity in both the cell lines (Fig. 4A and B). When the putative Pax2 motif was mutated in the whole promoter construct, a significant decrease ($P < 0.05$) in the promoter activity was found in both the cell lines (Fig. 4A and B). There was an appreciable decrease in the promoter activity of both the constructs with mutated Pax2 site in both the cell lines (Fig. 4A and B) indicating that the presence of Pax2 motif is important for the *wnt5* promoter activity. Co-transfection assays were performed with wild or mutant (for Pax2 site) *wnt5* promoter construct with Pax2 expression plasmids (pcDNA-Pax2Fr) to further confirm the role of transcription factor Pax2 in the activation of *wnt5* promoter. The promoter activity was upregulated significantly ($P < 0.05$) both in HEK 293 and CHO cells (Fig. 4C), when recombinant expression vector (pcDNA)

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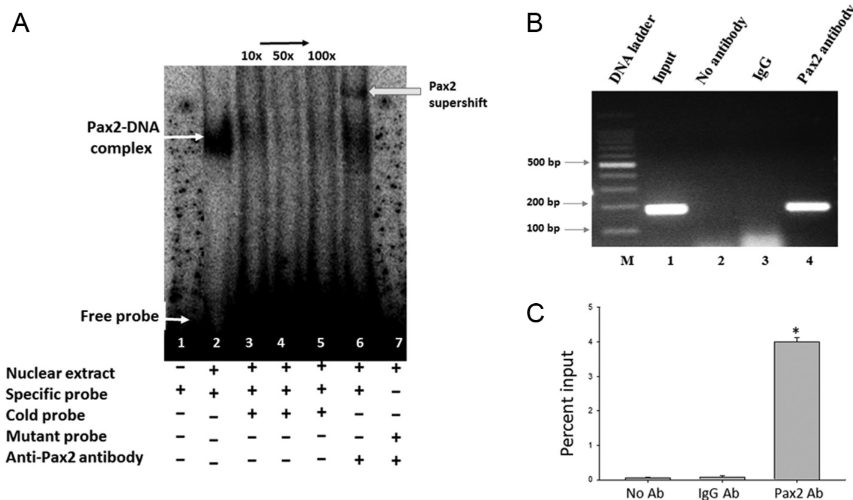

Figure 5
(A) EMSA with cold competition and supershift. Gel shift assay showing the binding of Pax2 (in the nuclear extract) to the oligomeric ^{32}P probe. Arrows indicate free probe, Pax2-DNA complex and the supershift observed upon the addition of Pax2 antibody. (B) Chromatin immunoprecipitation assay. Amplification of the *wnt5* promoter was positive only in the chromatin immunoprecipitated with Pax2 antibody. No signal was detected either with the control IgG or no antibody control. Input DNA was used as loading control. (C) qRT-PCR of immunoprecipitated samples. For qRT-PCR, each ChIP DNA fraction Ct value was normalized to the input DNA (1/10th of DNA used in ChIP assays) fraction Ct value. % input was calculated for each ChIP fraction and the values were plotted. The data represent the mean ± s.e.m. from three independent experiments.

with Pax2 coding sequence was co-transfected with the whole promoter construct and with Pax2 site. The promoter activity did not alter in both the cell lines when the recombinant expression vector (pcDNA-Pax2Rv) with Pax2 non-coding sequence was used for co-transfection as control (Fig. 4C). In the over-expression studies carried out with DC2 and DC3 constructs, promoter activity upregulated appreciably where the Pax2 sites are functional in both the cell lines compared with the constructs without pc-Pax2 supplementation. It shows the specificity of the transcription factor, Pax2, for the binding motif on the *wnt5* promoter.

Confirmation of Pax2 binding to *wnt5* promoter by EMSA and ChIP

To confirm the Pax2 binding, *in vitro* and *in vivo*, EMSA, supershift and ChIP analyses were performed. EMSA supershift was carried out using both normal and mutated Pax2-binding motifs of the *wnt5* promoter as probes. EMSA with catfish ovarian nuclear extracts demonstrated a DNA–protein complex formation (Fig. 5A) with ^{32}P end labeled-ds oligomeric probe containing normal Pax2 motifs (lane no. 2, Fig. 5A). Dissolution of binding (in the lanes 3–5, Fig. 5A) due to increasing concentration of cold competitor ds oligomer indicates the specificity of probe binding. Further, Pax2 antibody addition resulted in the supershift of protein–DNA complex (lane no. 6, Fig. 5A) mildly, whereas no binding was observed in the lane with mutated probe (lane no. 7, Fig. 5A). The direct interaction of Pax2 to its respective binding site was further verified using ChIP (Fig. 5B). With primers specific for the promoter of *wnt5*, intense PCR amplification was observed when the chromatin was immunoprecipitated with Pax2 antibody. No amplification was observed in no antibody control and when anti-IgG was used. Real-time analysis of percent input calculated showed high binding activity for Pax2 (Fig. 5C).

Discussion

The present report demonstrated the predominance of *wnt4* and *wnt5* during ovarian development and reproductive cycle of catfish. Transcript localization and hCG induction, *in vivo*, revealed their prominence in ovary and gonadotropin dependency. Further, the transcriptional interactions of *wnt5* with transcription factor, Pax2, in teleosts were demonstrated through functional promoter analysis of EMSA and ChIP using catfish ovarian nuclear extracts.

The deduced amino acid sequences of Wnt4 and Wnt5 possess the characteristic Wnt domain with N-terminal signal peptide that mediates protein secretion, 24 conserved cysteine residues for proper protein folding and N-terminal glycosylation and palmitoylation sites as Wnt solubilizing factors as that of other species. They shared high homology in conserved domains with other vertebrates suggesting their functional conservation (Chen *et al.* 2015). In most of the other teleost species, two paralogs of these genes were found and reported that teleost-specific whole genome duplication (TS-WGD) resulted in an extra set of paralogs for all fish genes (Christoffels *et al.* 2004), and over the period of evolution, 70% of these paralogs have been eliminated or inactivated leaving potential extra paralogs for every third gene in the teleost genomes (Wittbrodt *et al.* 1998). There are no other paralogs of these signaling molecules found in this

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species, which were conserved in other teleosts. This is particularly evident from the repeated RACE attempts at 5′ and 3′ ends and also through genomic DNA PCR with the primers spanning highly conserved exons and a variable intron. The absence of other paralogs in this species might have been due to its annual breeding pattern where the environment has much influences on its development, as it is proposed that teleosts have diversification potential during phases of environmental change (Glasauer & Neuhauss 2014) owing to TS-WGD. The phylogenetic analysis with the available deduced amino acid sequences of the other teleosts grouped them into the clade with Wnt4a and Wnt5a, respectively, for Wnt4 and Wnt5 affirming that their Wnt domain amino acid sequences are conserved throughout evolution (Yokoi *et al.* 2003).

Tissue distribution pattern of *wnt5* and *wnt4* showed their abundance in the ovary indicating a potential influence of these correlates as observed in other species (Vainio *et al.* 1991, Oreal *et al.* 2002, Pailhoux *et al.* 2002, Jaaskelainen *et al.* 2010), and in contrast to these, ovary-predominant function was not observed in rainbow trout (Nicol *et al.* 2012). The levels of *wnt4*, which seems to be homologous to its paralog, *wnt4a* observed in other species shows contradictory expression pattern by being predominant in ovary unlike *Cynoglossus semilaevis wnt4a* (Hu *et al.* 2014). Although the levels of expression are comparatively less in tissues other than ovary and kidney, the presence of *wnt* transcripts suggests their role in development as that of other teleosts (Matsui *et al.* 2005). This expression coincides with the ontogeny studies where their expression is apparent from the 0 dph indicating that they are one of the early developmental genes involved in embryogenesis (Ungar *et al.* 1995) and dimorphic pattern at 50 dph in favor of females, the critical period for sex differentiation of catfish, indicating their significance during sexual development like the observations in zebrafish (Sreenivasan *et al.* 2014). The dimorphism is maintained till adult stages indicating *wnt5* along with *wnt4* function at later stages of oocyte development and maturation as detected in mammals and black porgy (Wu & Chang 2009, Boyer *et al.* 2010*b*, Prunskaite-Hyyryläinen *et al.* 2014). Analysis of *wnt4* expression during ovarian reproductive cycle indicates its prominence in pre-spawning phase, which is contrary to the expression of *wnt5* indicating that both act in a cohesive way for ovarian growth as proved in mammals, with double knockouts of Wnt4 and Wnt5a that their coordinated signaling is essential for ovarian follicular development (Naillat *et al.* 2010). The localization of the transcripts and proteins of Wnt4 showed their presence in peri-nucleolar oocytes followed by pre-vitellogenic and vitellogenic oocytes indicating their plausible role in follicular development as that found in mammals (Boyer *et al.* 2010*b*). The upregulation of Wnt molecules by hCG as that of Wnt ligands in *wnt5b* and *wnt7b* in mouse mammary gland (Kuorelahti *et al.* 2007) indicates a stimulatory influence of gonadotropin on ovarian Wnts that might enhance ovarian growth, though their precise nature of hormonal mechanism is still largely unclear. Taken together, data from this study indicate that the signaling molecules, *wnt4* and *wnt5*, might have an important role in ovarian development and recrudescence of catfish, and their coordinated signaling may possibly trigger follicular development during the reproductive cycle with regulatory input from gonadotropins.

Synchronous expression of *pax2* and *wnt5* but not with *wnt4* during ovarian development, recrudescence, and spawning of catfish (present study, Prathibha & Senthilkumaran 2016) warrants promoter analysis of *wnt5* with a detailed examination of binding motifs, focusing on the transcription factor, Pax2. Although the promoter motif analysis can be extended to *wnt4*, our aim to limit it to *wnt5* is mainly due to lack of information on the transcriptional regulation of the latter when compared with the former in mammals. Promoter analysis of *wnt5* revealed binding sites for Pax2, as well as FOXO1, GATA1/6, Sox9b and CEBP. Among these motifs, Pax2 seems to be dominant, whereas others gave limited additive effects. Earlier studies revealed a role for *sox9b* during ovarian development in catfish (Raghuveer & Senthilkumaran 2010), probably having direct or indirect interactions. GATA 1/6, a family of transcription factors, which have been shown to be important in the regulation of genes directing differentiation in multiple organs (Viger *et al.* 2008), might be having interactions with Wnt pathway for ovarian development. FOXO1 is member of forkhead box O family of transcription factors that are known to regulate majority of follicle response genes in ovarian granulosa cells (Herndon *et al.* 2016), and it may probably operate through Wnt pathway. CCAAT-enhancer-binding proteins (or C/EBPs), a family of transcription factors, (Ramji & Foka 2002) which have been shown to be important in the regulation of genes directing differentiation in multiple organs, seem to play a role in ovarian development through Wnt.

Pax2, a member of paired box family of transcription factors, regulates developmental events in all vertebrates. In teleosts, its requirement is evident for the development

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of thyroid follicles in zebrafish (Wendl *et al.* 2002), which suggests probable role in ovarian development as impairment of thyroidal function differentially impairs gonadal development and recrudescence (Swapna *et al.* 2006, Swapna & Senthilkumaran 2007). In addition, the involvement of PAX2 in the development of mammalian urogenital system is relatively well known as it interacts directly with *WNT4* and *WNT5A* at the promoter level (Torban *et al.* 2006, Tamimi *et al.* 2008). However, no evidence exists in the recent past implicating a role for Pax2 in teleost ovarian development. In view of this, we analyzed the role of Pax2 during ovarian development and recrudescence of catfish by transient silencing studies *in vitro* and *in vivo*, which decreased the transcript levels of *wnt4* and *wnt5* to ~50% indicating direct or indirect interactions of these molecules with Pax2 (Submitted, Prathibha & Senthilkumaran 2016). In addition, the expression of transcripts encoding steroidogenic enzymes and certain transcription factors were also reduced indicating probable regulation of Pax2 on ovarian steroidogenesis through *wnt4/5* along with *ad4bp/sf-1*. Due to lack of information on transcriptional interaction of Pax2 on *wnt5*, in the present report, a series of experiments were performed to authenticate our findings.

At first, to evaluate the importance of Pax2 in teleosts, transient transfection studies were performed using two different cell lines, HEK293 and CHO. Luciferase reporter assays of progressive PCR deletion constructs showed higher promoter activity in the constructs having Pax2 motif compared with basal promoter activity in others. The site-directed substitution mutagenesis experiments of Pax2 motif on *wnt5* gene decreased the promoter activity of the constructs with Pax2-binding site compared with their wild constructs. Overexpression studies using Pax2 recombinant protein on these constructs demonstrated transcriptional interaction.

EMSA using catfish ovarian nuclear extract resulted in the formation of a Pax2–DNA complex with Pax2-specific oligomer probe. Further, a supershift was observed in the lane upon addition of anti-Pax2 antibody, which was absent with the mutated probe demonstrating a direct interaction of Pax2 on *wnt5* promoter, which is further validated by ChIP analysis. PCR carried out with DNA extracted from the Pax2 pull-down chromatin using specific primers of *wnt5* promoter resulted in a specific band with putative binding motif of Pax2, which is absent in DNA extracted from IgG pull-down chromatin and no antibody controls. qPCR analysis was used to further validate percent input that showed higher levels of interaction with Pax2 antibody, demonstrating the direct interaction of Pax2 on *wnt5* gene. Taken together, SDM, EMSA and ChIP analyses established a direct transcriptional interaction of Pax2 on *wnt5* gene, which might play an essential role in ovarian development and recrudescence of catfish. Although the Wnt signaling pathway that is operated by Wnt5 for its function is not clear yet, it appears that Wnt5 may interact with Wnt4 for ovarian follicle development as seen in mammals by Naillat *et al.* (2010) through double knock outs. However, in the Nile tilapia, a canonical signaling for ovarian development (Wu *et al.* 2016) has been reported. It remains to be tested further in light of our work and other reports from mammals. Nevertheless, present report for the first time identifies a transcriptional interaction of Pax2 on *wnt5* gene in a lower vertebrate signifying its importance in female reproductive system.

Conclusions

This study examined the expression of members of Wnt signaling molecules, Wnt4 and Wnt5, during ovarian development from the early stages of gonadal differentiation to follicular development till maturation in catfish. Ontogeny, phase-wise expression and gonadotropin regulation using hCG induction indicate a potential role for these correlates in ovarian development and recrudescence of catfish. Transcriptional interactional studies revealed that the involvement of *wnt5* in these processes is directly regulated by Pax2.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-16-0104.

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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