Activin receptor TypeIIB in rohu (*Labeo rohita*): Molecular characterization, tissue distribution and Immunohistochemical localization during different stages of gonadal maturation

Siddhi Patnaik
Duke-NUS Medical School
Lakshman Sahoo
ICAR-Central institute of Freshwater Aquaculture
Mausumee Mohanty
Barcode Biosciences
Amrita Bit
ICAR-Central Institute of Freshwater Aquaculture
Prem Kumar Meher
ICAR-Central institute of Freshwater Aquaculture
Sachidananda Das
Utkal University
Pallipuram Jayasankar
ICAR-Central Marine Fisheries Research Institute
Jatindra Nath Saha
ICAR-Central Institute of Freshwater Aquaculture
Paramananda Das (pdas77@hotmail.com)
ICAR-CIFA

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Abstract

Activin receptor type IIB (ActRIIB) is a transmembrane serine/threonine kinase receptor which plays a pivotal role in regulating reproduction in vertebrates including teleost. Earlier studies have documented its importance in governing gonadal maturation in higher vertebrates. However, reports on regulation of fish reproductive system by ActRIIB gene are still limited. Here, we report identification and characterization of *ActRIIB* cDNA of *Labeo rohita*, a commercially important fish species of the Indian subcontinent. The full-length gene encoding rohu *ActRIIB* was cloned and found to be of 1674 bp in length. Functional similarities were evident form evolutionary analysis across vertebrates. Real-time PCR to measure the expression of *ActRIIB* transcript in rohu revealed significant mRNA levels in gonads followed by non-reproductive tissues, including brain, pituitary and muscle. With respect to different gonadal maturation stages, predominant expression of *ActRIIB* mRNA was observed during the pre-spawning phase of both sexes. To further delineate its role in rohu reproduction, a recombinant protein of the extracellular domain of *ActRIIB* (rECD-ActRIIB) was produced and polyclonal antibody raised against the protein for its immuno-localization studies during different gonadal maturation stages. Strong immunoreactivity was noticed in the pre-vitellogenic oocytes which decreased dramatically in the fully mature oocytes. Similarly, the strong and intense immunoreactivity was found in the spermatids and spermatocytes of the immature testis, and eventually the intensity reduced with the progression of the maturation stage. These results provide the first evidence of presence of *ActRIIB* in rohu gonadal tissues. Taken together, our observations lay the groundwork for further understanding and investigation on the potential role of *ActRIIB* in fish reproduction system in the event of gonadal maturation.

Introduction

As aquaculture industry aims to boost production of viable eggs with high survival rate, it is vital to bridge the gaps that still remain in understanding the dynamic processes controlling teleost reproduction. Regulation of oocyte maturation and spermatogenesis in fish is a complex process that involves interaction of several regulatory molecules. Activin receptors (ActR*s*), members of transforming growth factor-beta (TGF-β) superfamily, with their unique characteristics of wide range ligand specificities are capable of governing several cellular responses in reproduction and development. They are single-pass transmembrane serine/threonine kinase receptors and are basically of two types: type I and type II, having a small extracellular ligand binding domain and an intracellular kinase domain, which act cooperatively to transduce signals for important physiological and molecular processes such as cell proliferation, growth and differentiation of skeletal tissues and gonadal function (Mathews et al., 1994; Mathews and Vale, 1991; Patnaik et al., 2017; Goebel et al., 2019). Multiple TGF- β ligands like activin, myostatin, follistatin, in their bio-active dimeric forms bind to the extracellular domain of the type II receptors for signal activation. Both the receptors undergo dimerization followed by phosphorylation of Gly-Ser residues in the GS domain preceding the kinase domain for signal transmission (Huang and Chen, 2012; Kubiczkova et al., 2012). Amongst the many ligands of TGF-β family, activin upon interaction with type II receptors plays a key role in promoting follicle growth, primarily regulating ovarian

maturation. Two variants of type II receptors such as IIA (*ActRIIA*) and IIB (*ActRIIB*) are known (Tsuchida et al., 1993; Yamashita et al., 1994; Dijke et al., 1996) having differences in binding affinity and signalling strength and produced by differential splicing. Experimental findings suggest that *ActRIIB* has higher affinity for activin than *ActRIIA* (Attisano et al., 1992). Despite their structural resemblances, no functional overlap or sequence similarity exist between the two receptors.

Till date, ActRs have been characterized in higher vertebrates and its importance in regulating vertebrate reproduction and development has also been studied. Biochemical and localization studies have demonstrated expression of *ActRII* mRNA in bovine and mouse testis (Ethier et al., 1994; Wu et al., 1994) suggesting their putative role in testis maturation. Experimental evidences of the presence of activin receptor and Smad proteins in oocytes of rat might implicate their role in follicular development (Drummond et al., 2002). Moreover, expression of *ActRIIA/IIB* has been found in oocytes of other vertebrates regulating both folliculogenesis and differentiation (Van den Hurk and Van de Pavert, 2001). Recently, it has been demonstrated that activin, TGF-β, Nodal and their respective type II and type I receptors are expressed in sex and cell type specific patterns suggesting their specific role in testis and germ cell development (Miles et al., 2013).

Among teleosts, genes encoding *ActRIIA* cDNA have been cloned and characterised in zebrafish and grass carp (Nagaso et al., 1999; DiMuccio et al., 2005). The significance of *ActRIIA* in zebra fish reproductive axis is evident from its expression in the oocyte during embryonic developmental stages (Nagaso et al., 1999). Rohu *ActRIIA* cDNA has been characterized and its expression during different reproductive stages studied (Patnaik et al., 2017). There are ample evidences that have supported specific role of *ActRIIB* in regulating skeletal muscle development in fishes (Carpio et al., 2009; Funkenstein et al., 2012). Although, most of the studies on *ActRIIB* are associated with its role in regulating body growth, there are also evidences that suggest its importance in regulating teleost reproduction and development. Experimental studies provide the proof of *ActRIIB* expression in zebrafish oocyte at different developmental stages (Garg et al., 1999; Wu et al., 2000). However, much less is known about the expression of *ActRIIB* in teleost gonad and its importance in ovary and testis maturation. Therefore, molecular characterization of activin receptors in large number of non-model fish species may be essential to establish molecular mechanisms underlying its role in fish reproduction.

Indian major carp, *Labeo rohita* (rohu), is a popular table fish widely cultured in monoculture and polyculture systems of India and adjacent countries. In the present study, we have identified and characterized ActRIIB to determine its role in rohu reproduction. Furthermore, expression of *ActRIIB* during different reproductive stages in gonadal tissue has also been detected and quantified. We have also synthesized a recombinant protein for extracellular domain of rohu *ActRIIB* (rECD-ActRIIB) and have produced polyclonal antibodies in order to investigate the immunohistochemical localization of the receptor gene in the gonadal tissue during the pre- and post- reproductive stages. These findings in future would help us to comprehend the importance of the receptor gene in regulating fish reproduction, particularly during gonadal maturation and development in a more defined way.

Materials And Methods

Sample preparation

Rohu carps collected from the farm of Indian Council of Agricultural Research (ICAR)-Central Institute of Freshwater Aquaculture, Bhubaneswar, India were used for this study. Fishes were anesthetized with 300 mg/L of tricaine methane sulfonate (MS222; Sigma–Aldrich) before collection of tissue samples. For studying tissue distribution, testis, ovary, kidney, heart, liver, intestine, brain, pituitary and muscle were dissected. For expression studies during different reproductive stages, tissue samples were collected from pre-spawning (March-May), spawning (June-August) and post-spawning (September-October) phases of rohu. All the samples were snap-frozen before storage in liquid nitrogen and RNA extraction.

RNA extraction and cloning of ActRIIB cDNA

Total RNA extraction and cDNA synthesis was performed as described earlier (Patnaik et al., 2017). In brief, RNA was extracted using TRIzoL reagent from approximately 100 mg to each tissue. Subsequently, DNA was removed by DNAse I treatment followed by column purification. Reverse transcription was performed taking ~ 5µg of total RNA from testis tissue, using SuperScript® III cDNA synthesis kit (Invitrogen, USA) as per the manufacturer's instructions. Partial cDNA sequence of rohu *ActRIIB* was amplified using first-strand cDNA as template. The PCR primers used for this purpose (Supplementary Table S1) were designed from conserved regions of the genes of related species available in the NCBI database (http://www.ncbi.nlm.nih.gov). The PCR amplification was carried out in 25 µl of reaction volume with following conditions: initial denaturation at 95°C for 5 min followed by 30 cycles with 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute and finally 72°C for 7 minutes. The amplified products were gel purified and cloned into pGEM-T Easy Vector (Promega, USA) and sequenced in ABI 3730 XL automated DNA analyzer (Perkin-Elmer Applied Bio system) using SP6 and T7 primers. Verification of partial *ActRIIB* sequences was done using NCBI- BLAST service.

Gene specific primers (Supplementary Table S1) were designed from the rohu partial ActRIIB sequence obtained above, and full-length cDNA of rohu *ActRIIB* was generated by Rapid Amplification of cDNA ends (RACE) with different primer sets using SMARTerTM RACE cDNA Amplification Kit (Clontech, USA) as described earlier (Patnaik et al., 2017). In brief, touch down PCR was carried out with different sets of 3' and 5' primers as follows: one cycle of initial denaturation at 94^0 C for 2 min followed by five cycles at 94°C for 30 s, 64°C for 3 min; five cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 3 min and another 27 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 3 min. Amplified products were cloned and sequenced as mentioned above.

Bioinformatic analysis

The full-length cDNA sequence generated was blasted against the public database using NCBI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The program ORF finder of NCBI (http://www.ncbi.nlm.nih.gov) was used for identification of putative amino acid translation and open reading frames (ORF) followed by

validation in EXPASY translate tool. Rohu ActRIIB amino acid sequence was aligned with other ActRIIB amino acid sequences using the program BioEdit v7 (Hall 1999). SignalP4.0 server (http://www.cbs.dtu.dk/services/SignalP-4.0/) was used to predict signal peptide of rohu ActRIIB. TMPred (http://www.ch.embnet.org/software/TMPRED) and Protter (http://wlab.ethz.ch/protter/start/) were used to predict transmembrane helixes. Further, InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan5/) and SMART program as implemented in ExPASy tools (http://smart.embl-heidelberg.de/) were used to characterize the motifs and domains present in rohu ActRIIB. ScanProsite (http://www.expasy.org/tools/scanprosite/), Protparam (http://web.expasy.org/protparam/) and NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhosK/) programs were used to predict the serine/threonine kinase active-site, to determine the physico-chemical properties and to detect phosphorylation sites, respectively. For prediction of secondary structures, SOPMA (http://nhjy.hzau.edu.cn/kech/swxxx/jakj/dianzi/Bioinf7/Expasy/Expasy8.htm) was used and PsiPred (http://bioinf.cs.ucl.ac.uk/psipred/) was used to assess the confidence level. A Neighbor-Joining phylogenetic tree was generated following MEGA 6.06 (Tamura et al., 2013) with bootstrapping of 1,000 repetitions. The program, Matrix Global Alignment Tool Program (Campanella et al., 2003) was used to deduce the similarity and identity percentage of rohu ActRIIB protein with others. Table 1 presents the accession numbers of protein sequences used in the present study.

Quantitative real-time PCR

Gonadal tissue expression pattern during different reproductive phases and tissue distribution study were carried out by quantitative real-time PCR (qRT-PCR) as described earlier (Patnaik et al., 2017) using Light Cycler$^{@}$ 480 II real-time PCR detection system (Roche, Germany). Reactions for expression studies were performed in triplicates using SYBR Green Real-time Master Mix II (Roche Diagnostics, Germany) at an annealing temperature of 58^{0} C. Possibilities of DNA contamination was excluded by taking negative control in each experiment, and determination of PCR efficiency was carried out by serial dilution. The house keeping gene *β-actin* was taken as a positive calibrator for normalization of target gene expression. The PCR primers used for gene expression study are presented in Supplementary Table S1. Melting curve analysis was performed to confirm primer specificity, and agarose gel electrophoresis was carried out to check size of transcript.

Expression of Recombinant Extracellular Domain of rohu ActRIIB *(rECD-ActRIIB)*

The recombinant protein for the extracellular domain was produced using pET28a expression vector. Rosetta DE3 host cells harbouring the recombinant plasmid, *pET28rECD-ActRIIB* was transformed and positive colony (Kanamycin & Chloramphenicol resistant) grown overnight in 3ml culture tube containing Luria Bertani (LB) broth following the manufacturer's standardized protocol. Thirty ml seed culture was grown by inoculating LB broth with 1% of overnight culture and kept in shaker at 37°C. The overnight grown seed culture was inoculated in 1 litre LB. When the culture attained an absorbance of 0.6 at 600 nm, the cells were induced with 1mM IPTG for a minimum period of 4 h at 37°C. Induced culture cells were harvested by centrifugation. The pellet obtained was washed in 20 ml of wash buffer (Tris-50mM,

NaCl- 300mM, pH-7.4, Stock concentration-100mg/ml). After washing, 0.5 mg/ml lysozyme (Stock-100mg/ml) was added and incubated at room temperature in a shaker at 37°C for 30 mins followed by sonication for 10 mins. The pellet obtained after centrifugation was again suspended in wash buffer and again incubated in shaker for another 30 mins. The suspension was then centrifuged and the clear supernatant was collected following protein purification.

Purification of Recombinant protein *(*rECD-ActRIIB*)*

1 ml Ni-NTA resin was packed in a BioRad column and washed by passing 10 cv de-ionized water. The column was equilibrated by passing 20 cv equilibration buffer (50mM Tris, pH 7.4, 8M Urea, 300mM NaCl). The inclusion body (prepared as per the standard protocol) was solubilized in 50mM Tris, pH 7.4, 8M Urea, 300mM NaCl containing 10 mM Imidazole. Sample was centrifuged and the supernatant obtained was loaded onto Ni-NTA resin and incubated for 1 hr. After incubation, the supernatant was allowed to pass and the column was washed by passing 10cv equilibration buffer with 10mM imidazole. On column, refolding was done by passing gradient from 8M Urea to 2 M urea in 50mM Tris, pH 7.4, 300mM NaCl (200 mL). Final wash was given with 10cv 2 M Urea, 50mM Tris, pH 7.4, 300mM NaCl, 25 & 50 mM imidazole. The protein was eluted with 10 cv elution buffer; 2 M Urea, 50mM Tris, pH 7.4, 150mM NaCl, 5% Glycerol, 500mM Imidazole. The eluted fractions were analyzed in SDS-PAGE and fraction containing pure proteins were pooled and dialyzed against buffer: 2 M Urea, 50mM Tris, pH 8, 150mM NaCl and 10% Glycerol. Protein was concentrated by using 3 kDa MWCO Centricon (Millipore) at 4000 rpm. The protein was kept in small aliquots at -80°C for further use.

SDS-PAGE Analysis

SDS-PAGE was carried out according to Laemmli (1970). Samples were mixed with loading buffer in the presence of 5% β-mercaptoethanol and separated in 12.5% mini gels. Protein bands were visualized by Coomassie Brilliant blue staining.

Polyclonal Antibody Production and Validation

Polyclonal Ab against the rECD-ActRIIB peptide of rohu was raised in two rabbits, A and B for immunohistochemical localization study in gonadal tissue of rohu during pre- and post-reproductive stages. The Ab raised was further validated by western blot and Indirect ELISA.

Western Blot Analysis

For antibody validation, cell lysate electrophoresed on 12% SDS-PAGE were transferred onto the nitrocellulose membrane in Western transfer buffer (25 mM Tris-Cl pH 8.3, 192 mM glycine and 10% methanol) using Bio-Rad Trans blot Apparatus following Towbin et al. (1979). Pre-immune sera and 3rd immune sera of both Rabbit A & B were tested on recombinant protein (20ng) at 1:50,000 dilutions. 5% blocking solution Skim Milk Powder in 1X TBST (pH: 8.0) and 1% SKM in TBST was prepared freshly. Antibodies were diluted in 1% blocking solution (~ 2.3µl in 5ml blocking solution) and shaken well for at least 15 mins. The strips were soaked in methanol for 2 mins. The strips were washed twice with 1X

TBST for 5 mins. The strips were blocked in 5% blocking solution for 15 min while shaking. The strips were washed 2 to 3 times in 1X TBST for 5–10 mins. The strips were transferred on the antibody solutions for primary Ab incubation while shaking for 2hrs at RT and then at 4°C. The next day, strips were washed once in 1X TBST for 30 mins and strip blocks were made. The blocks were washed in 1X TBST for 30–45 mins with twice change of TBST. TBST was discarded and secondary Ab (HRP-conjugate Anti-Rabbit IgG) (1:5000 dilution in 1% blocking solution) was added and incubated for exactly 1hr at RT. The blocks were washed in 1X TBST for 2–3 hrs with 3 to 4 changes to reduce background staining. Chemiluminiscence's solution (SuperSignal WestPico™ Chemiluminiscent, Pierce), 1:1 solution A and solution B was prepared and kept for 5 mins and was mixed well and kept for 5mins. The strip blocks were soaked well in the solution for 5 mins under constant shaking. The strip blocks were arranged on a Hyper film with fluorescent marks on the corner and transparent film was placed over it. Three exposures at 15sec, 1.5min and 3min were taken for observation of the band development pattern. The film was taken out and kept in developer solution and allowed to develop followed by washing in tap water and kept in fixer solution. After 5 to 10 mins, the film was taken out and then washed in running water and the film was dried. Finally, the developed film along with the strips was aligned on the fluorescent paper and individual lanes and marker was marked.

Indirect ELISA analysis

Indirect ELISA was performed from the immune sera obtained from the immunized rabbit A & B to validate the polyclonal Ab produced. First and third immune sera of Rabbit A were tested against the synthesized antigen at 200 ng, at 1:5000 dilution of primary Ab. Similarly, first and third immune sera of Rabbit B were tested against synthesized antigen at 200 ng, at 1:5000 dilution of primary Ab. Pre-immune sera were used as control in place of primary Ab in both Rabbit A and B. Plates read were taken after 15 min of enzyme substrate reaction.

Immunohistochemistry

Rohu testis and ovary collected from immature pre-reproductive and mature reproductive stages were cut into small sections and preserved in 10% NBF (neutral buffer formalin) for 18–24 hours. Paraffin-wax embedded tissue blocks made were cut into serial sections in LEICA RM2125 microtome to the desired thickness of 3 microns and mounted to microscopic slides treated with Chicken/Mayer's albumin and kept in room temperature for overnight. The slides were then deparaffinized and rehydrated by routine protocol. The slides were suspended in 3% H_2O_2 diluted in methanol and kept in humid chamber for 15 mins. Heat-induced antigen retrieval was performed using the Tris-EDTA Buffer (pH 9.0) in a microwave oven for 3 mins. The slides were washed three times 3 minutes each with 1X TBST buffer and kept in slide jar with warm Tris-EDTA buffer for antigen retrieval for 15mins at room temperature followed by again washing. The slides were blocked in boxes by Pap pen marker and then suspended in Sniper (Biocare Medical, USA) for 15mins in humid chamber in order to block and minimize background staining, The slides were washed in 1X TBST buffer and the sections were suspended with primary antibody (polyclonal Ab raised against rECD-ActRIIB) in three dilution ratios, i.e., 1:500, 1:750 and 1:1500

(dilution in 1X PBS) and incubated for 90mins in a humidified chamber. The slides were rinsed with 1X TBST and then suspended in HRP-conjugated secondary antibody (UltraTek Anti-polyvalent Biotinylated Ab) for 10mins in humidified chamber followed by washing in 1X TBST buffer. Three hundred microliter substrate buffer (Betazoid DAB chromogen) was applied for 10 minutes to stain the nuclei of the cells in the sections. Then haematoxylin was added and the slides were washed under tap water followed by rehydration in xylene for 20-30mins. Slides were mounted with DPX and the sections were observed for immunostaining using the LEICA light microscope with digital camera. As control, primary antibodies were omitted in a few test sections in each experiment.

Data analysis

Gene expression data obtained from qRT-PCR study in triplicates, along with β-actin was used to normalize any differences in reverse transcriptase efficiency. Relative gene expression was calculated using Pfaffl method (Pfaffl 2001) based on the threshold cycle (C_t) value. All data are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Duncan multiple-range test using SPSS18.0 software.

Results

Molecular Cloning and sequence analysis of rohu *ActRIIB*

Labeo rohita ActRIIB partial cDNA (*LrActRIIB*) was synthesized from testis RNA using normal PCR that generated a sequence of 939 bp. The complete CDS of *LrActRIIB* was obtained by RACE PCR strategy that produced a sequence of 1674 bp in length (**KX710215**) with 27 bp of 5' UTR and 114 bp of 3'UTR (Fig. 1). The ORF consisted of 1533 bp that translated into 510aa sequence with a molecular weight of 57.7 KDa and theoretical isoelectric point of 5.43. Comparison of *LrActRIIB* with teleost, amphibian, avian and mammalian orthologs exhibited similarity ranging from 94–75% showing highest similarity with fishes (data not shown). Amino acid sequence alignment of *LrActRIIB* with other vertebrate counterparts revealed that the gene is organized into three major domains, typical of TGF-β superfamily, including a signal peptide (SP, 1-22aa), a short extracellular domain (ECD, 27-117aa), a hydrophobic transmembrane domain (TMD, 136-158aa) and a large intracellular serine/threonine kinase domain (ICD, 188-476aa). Disulphide bonds formed by the 10 conserved cysteine residues present in rohu ActRIIB govern the folding of the ligand-binding domain (Fig. 1) which is in agreement with earlier reports (Greenwald et al., 1999; Lin et al., 2006; Walton et al., 2012). The characteristic HRDFKSKN (317-324aa) and GTRRYM (358-363aa) Ser/Thr kinase motifs present in the ICD region and $D_{319,}$ respectively are predicted to be the Ser/Thr kinase active-sites. The characteristic ESSI motif essential for mediating functions between ActRIIs and PDZ-domain of activin receptor-interacting proteins (ARIPS) (Tsuchida et al., 2004) is present at the C-terminal end of *LrActRIIB*. Further, it is predicted that KARGRFGCV (194-202aa) and K_{215} are nucleotide binding site and ATP-binding site, respectively. Two potential N-glycosylation sites and potential post-translational modification (PTM) sites are also found to be present in the putative transmembrane structure of the rohu ActRIIB. It is predicted to have 17 serine phosphorylation sites, 7

threonine phosphorylation sites and 4 tyrosine phosphorylation sites. Secondary structure analysis using SOPMA showed that *LrActRIIB* holds 43.33% alpha helix, 36.67% random coil, 13.98% extended strands and 6.02% beta turns.

Phylogenetic Analysis

The evolutionary relationship of rohu gene with other vertebrates was evaluated by constructing a Neighbor-Joining (NJ) tree using the protein sequences available in the public database. The tree showed two distinct clades, with teleost ActRIIBs forming one major cluster whereas higher vertebrates, i.e., mammalian ActRIIBs forming another major cluster as illustrated in Fig. 2. The teleostean clade was further sub-divided according to their habitat and family. Percentage of similarity and identity of rohu ActRIIB amino acid sequences with other species are presented in Table 1. Highest percentage of similarity and identity (97.4% and 95% respectively) were observed between *L. rohta* and *C. idella* demonstrating their high degree of relatedness.

Quantitative expression of ActRIIA mRNA during different reproductive and growth stages

Tissue distribution of ActRIIB mRNA

qRT-PCR analysis revealed the expression of *ActRIIB* transcript in different tissues of juvenile rohu. Expression of *ActRIIB* mRNA was observed in all tissues studied. Highest expression was found in gonad then brain, pituitary, liver and muscle. In contrast, expression in intestine, heart and kidney is exceptionally low. The expression data of rohu *LrActRIIB* is depicted in Fig. 3A with P value < 0.05.

Expression profiling of *LrActRIIB* mRNA during different reproductive stages of rohu

Variation in expression pattern during pre-spawning, spawning and post-spawning stages was carried out. During pre-spawning phase expression of *LrActRIIB* transcript in ovary observed to be highest. Similarly, in testis during pre-spawning phase *LrActRIIB* expression was significantly high followed by a remarkable decline during spawning and post-spawning stages. Expression profile of *LrActRIIB* in the gonadal tissue during different reproductive stages of rohu ($P < 0.05$) is presented in Fig. 3B.

Expression and Purification of recombinant extracellular domain of LrActRIIB protein *(*rECD-ActRIIB*)*

The recombinant protein, i.e., extracellular domain of *LrActRIIB* of 113aa was synthesized and expressed in pET28a vector. The theoretical molecular weight and pI was estimated to be 14.04 kDa and 4.73 respectively. For the screening of the expressed protein, the supernatant and the pellet isolated from induced cells were run on SDS-PAGE and the protein of approximately similar molecular weight to the estimated value was found in the supernatant as shown in Fig. 4A. The isolated protein was purified on Ni-NTA column and on column refolding was done by passing gradient from 8M Urea to 2 M urea in 50mM Tris, pH 7.4, 300mM NaCl. After the final wash, the protein was eluted with 10 cv elution buffer; 2 M Urea, 50mM Tris, pH 7.4, 150mM NaCl, 5% Glycerol, 500mM Imidazole and fractions containing pure

protein were pooled and dialyzed against buffer: 2 M Urea, 50mM Tris, pH 8, 150mM NaCl and 10% Glycerol as shown in Fig. 4B.

Production and Validation of Polyclonal Antibody against rECD-ActRIIB peptide

Polyclonal Ab against the rECD-ActRIIB peptide was produced for immunohistochemical study in gonadal tissue of rohu during pre-spawning and spawning stages. The Ab raised was further validated by western blot analyses of the immune sera derived from the two immunized rabbit, resulting in a single band in the blot with an apparent molecular weight slightly higher than that of the calculated weight of ~ 14 kDa as shown in Fig. 5. Besides western blot, Indirect ELISA was also performed from the immune sera obtained from the immunized rabbit A & B to validate the polyclonal Ab produced. First immune sera of Rabbit A tested against the synthesized antigen at 200 ng, at 1:5000 dilution of primary Ab obtained a value of 1.176 OD at 450 nm. Third immune sera of Rabbit A, also tested against synthesized antigen at 200 ng, at 1:5000 dilution of primary Ab obtained a value of 1.215 OD at 450nm. Similarly, first immune sera of Rabbit B, tested against synthesized antigen at 200 ng, at 1:5000 dilution of primary Ab obtained a value of 1.056 OD at 450 nm. Third immune sera of Rabbit B, tested against synthesized antigen at 200 ng, at 1:5000 dilution of primary Ab obtained a value of 1.174 OD at 450 nm as shown in Fig. 6A. Third immune sera of Rabbit A were purified and validated by Indirect ELISA. Protein A Purified Ab (200 ng), tested against 200 ng of antigen, obtained a value of 2.572 OD at 450 nm as shown in Fig. 6B. Pre-immune sera were used as control in place of primary Ab at 1:5000 dilutions. The purified Ab was further used for immunohistochemical localization study in rohu gonadal tissue during reproductive stages.

Immunohistochemical localization study in gonadal tissue of rohu during pre-spawning and spawning stages

In the previtellogenic ovary, the cytoplasm of oogonia and primary oocytes displayed strong immunoreactivity of ECD-ActRIIB (Fig. 7A.i-ii). As ovarian maturity progressed, follicle cells (granulosa) surrounding the oocyte showed weak immunoreactivity. Also, specific immunostaining in the follicular cell layer of vitellogenic oocytes, but the nuclei (germinal vesicles) were completely negative with no staining (Figs. 7A iv-vi). In addition, the cytoplasm of oocytes that started to accumulate yolk inclusions gradually lost its immunoreactivity, with the reaction intensity being inversely proportional to the amount of yolk globules in the oocyte. The oocytoplasm of mature oocyte was weak for ActRIIB whereas immunostain was found in zona pellucida region and follicular cell layer. The ECD-ActRIIB Ab immunoreactivity in the pre-vitellogenic stage was stronger and the immunostain reduced with maturation of oocyte. In immature male rohu during spermatogenesis in pre-spawning stage, the testis was filled with primary and secondary spermatocyte and spermatids, showing strong immunoreactivity to ECD-ActRIIB Ab. However, during spawning stage the testis was filled with spermatozoa and was in the stage of spermiating. The immunoreactivity reduced with maturation and the germ cells in the stage of spermiation showed weak immunostain than pre-spawning stage as noticed in a similar pattern in the oocytes (Fig. 7B i-vi)

Discussion

In the present study, *ActRIIB* of *Labeo rohita* has been cloned and characterized. ActRIIB cDNA of *L. rohita* consists of 510 aa with 89–95% similarity to its vertebrate counterparts. The characterized protein in rohu possesses the typical domain architecture of TGF-β type II receptors with conserved cysteine residues in the activin receptor-specific extracellular domain essential for ligand binding (Kingsley, 1994; Josso et al., 1997; Shav-Tal et al., 2001). Moreover, Ser/Thr kinase motifs in ICD appeared to be well conserved. The protein sequence, "KARGRFGCV" for nucleotide binding is conserved for type II receptors of TGF- β superfamily. This is striking to observe such high level of structural conservation through evolution, suggesting similar functional role of the gene in fishes including rohu. The PDZ motif essential for receptor binding to PDZ protein for interaction with ARIPS for receptor internalization is conserved for type II receptors. The COOH-terminal sequence of rohu ActRIIA is E-S-S-L (Patnaik et al., 2017), and the COOH-terminal sequence of *LrActRIIB* is E-S-S-I, both of which are concordant with a class I consensus PDZ binding motif XSX (V/I/L) (where X is any amino acid). Among the various receptor, ActRIIs from different species contain a PDZ binding motif at their COOH-terminus (Shoji et al., 2000; Matsuzaki et al., 2002; Tsuchida et al., 2004). The structural dissimilarity might suggest difference in their receptor functioning upon different ligand interaction. Phylogenetic analysis showed that the receptor gene is evolutionarily conserved across the vertebrate lineage signifying that it might share common biological functions with its other counterparts. *LrActRIIB* formed a major cluster with teleosts depending on their habitat. High degree of similarity/identity value observed between *L. rohita* and its counterparts may suggest structural and functional similarity among them.

Functional involvement of *ActRIIB* in diverse biological processes was evident form ubiquitously expression in reproductive as well as non-reproductive tissues. However, high expression level of the gene in gonad, brain, pituitary, muscle and liver might implicate its potential role in governing physiological traits like reproduction and body growth. Recently, we have also examined the expression of *ActRIIA* gene in rohu demonstrating its importance in regulating gonadal maturation and skeletal muscle development (Patnaik et al., 2017). The results of the present study are also in agreement with previous reports in other vertebrates including teleosts (Carpio et al., 2009; Otto and Patel, 2010; Østbye et al., 2007). A comparative expression profiling of the transcript has been done in the gonadal tissue of rohu during the reproductive cycle, i.e., pre-spawning, spawning and post-spawning stages. In agreement with previous studies, high level of expression of rohu *ActRIIB* mRNA in gonad during pre-spawning stage demonstrated its significant role in stimulating oocyte maturation and spermatogenesis (Wu et al., 2000; Van den Hurk and Van de Pavert, 2001; Pangas et al., 2002; Silva et al., 2004). However, decreasing *LrActRIIB* transcript expression towards the post-spawning stage might suggest the effect of feedback inhibition of follicular activin signaling by antagonists like inhibin to decrease FSH release from pituitary as described in earlier reports (Yuen and Ge, 2004; Cheng et al., 2007).

Besides qRT-PCR studies showing high expression levels of the receptor gene in gonadal tissue, we also determined the spatial distribution of the immunoreactive ECD-ActRIIB peptide in ovary and testis of rohu by immunohistochemical study. In the previtellogenic and vitellogenic ovary of *L. rohita*, the cytoplasm of

oogonia and primary oocytes exhibited strong immunoreactivity of ActRIIB peptide. However, the immunoreactivity significantly decreased and was eventually found to be localized in the follicular cell layer and zona pellucid region when the oocytes became fully mature. This result signify its role in regulating oocyte maturation as reported in earlier studies (Wu et al., 2000, Cheng et al., 2007). The decrease in intensity of immunoreactivity suggests that *ActRIIB* mRNA expression may decrease with the development of oocytes. Alternatively, this could be due to the dilution of a constant amount of the mRNA as oocytes become larger. This result is consistent with the reports of previous studies in zebrafish (Garg et al., 1999) as well as in mammals (Cameron et al., 1994). In the localization studies of ECD-ActRIIB peptide in male rohu, strong and intense immunoreactivity was found in spermatids and spermatocytes of immature testis. However, in mature rohu immunosignals was particularly evident towards the periphery of the testis, but with reduced immunostaining centrally. This result is in accordance with previous studies as reported in mammals (Anderson et al., 2002) suggesting its role in regulating spermatogenesis in rohu. In correlation with the present localization study of ECD-ActRIIB peptide in rohu, *ActRII* and *ActRIIB* mRNA have also been detected in bovine and rat (Cameron et al., 1994; Ethier et al., 1994; Drummond et al., 2002). Thus, we can conclude that the receptor gene might possess potential role in regulating reproductive physiology of rohu.

Conclusion

The present study reports for the first time the characterization of ActRIIB gene in *L. rohita* and its spatial and stage-specific expression during different reproductive phases. Furthermore, recombinant protein, *Lr-ActRIIB-ECD* found to be localized in the gonadal tissues of rohu provides the evidence of its potential in promoting oocyte maturation and spermatogenesis. Thus, the information obtained from this study will be of interest for further research towards understanding great details of ActRIIB involvement in teleost reproductive system.

Declarations

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Declarations

Funding: Not Applicable

Conflicts of interest: The authors declare that they have no conflicts of interest.

Ethics approval: All handling of fish was carried out following the guidelines for control and supervision of experiments on animals by the Government of India and approved by Institutional Animal Ethics Committee (AEC) of ICAR-CIFA.

Consent to participate: Not Applicable

Availability of data and material: The *ActRIIB* cDNA sequence was submitted in the GenBank with accession number **KX710215**.

Authors' contributions: PD, SD and PJ conceived the project; JNS performed animal rearing and tissue sampling; SP, MM & LS carried out the laboratory experiments; AB & PKM carried out the bioinformatics analysis; SP, LS, PD, SD & PJ wrote and reviewed the MS. All authors read and approved the manuscript.

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Tables

Table 1: Percentage of similarity and identity of *Labeo rohita* ActRIIA with other species counterparts

Species	Similarity (%)	Identity (%)
Danio rerio NP_001103748.2	95.8	93.8
Ctenopharyngodon idella FJ686114.1	99.1	97.0
Dicentrarchus labrax HE967321	90.9	81.2
Xenopus laevis NP_001084061	89.4	77.6
Gallus gallus NP_990698.1	90.7	80.9
Bos taurus NP_776652.1	89.2	78.0
Rattus norvegicus NM_031571.2	89.0	77.5
Mus musculus NM_007396.4	89.0	77.7
Homo sapiens AAH67417.1	88.8	77.5

Figures

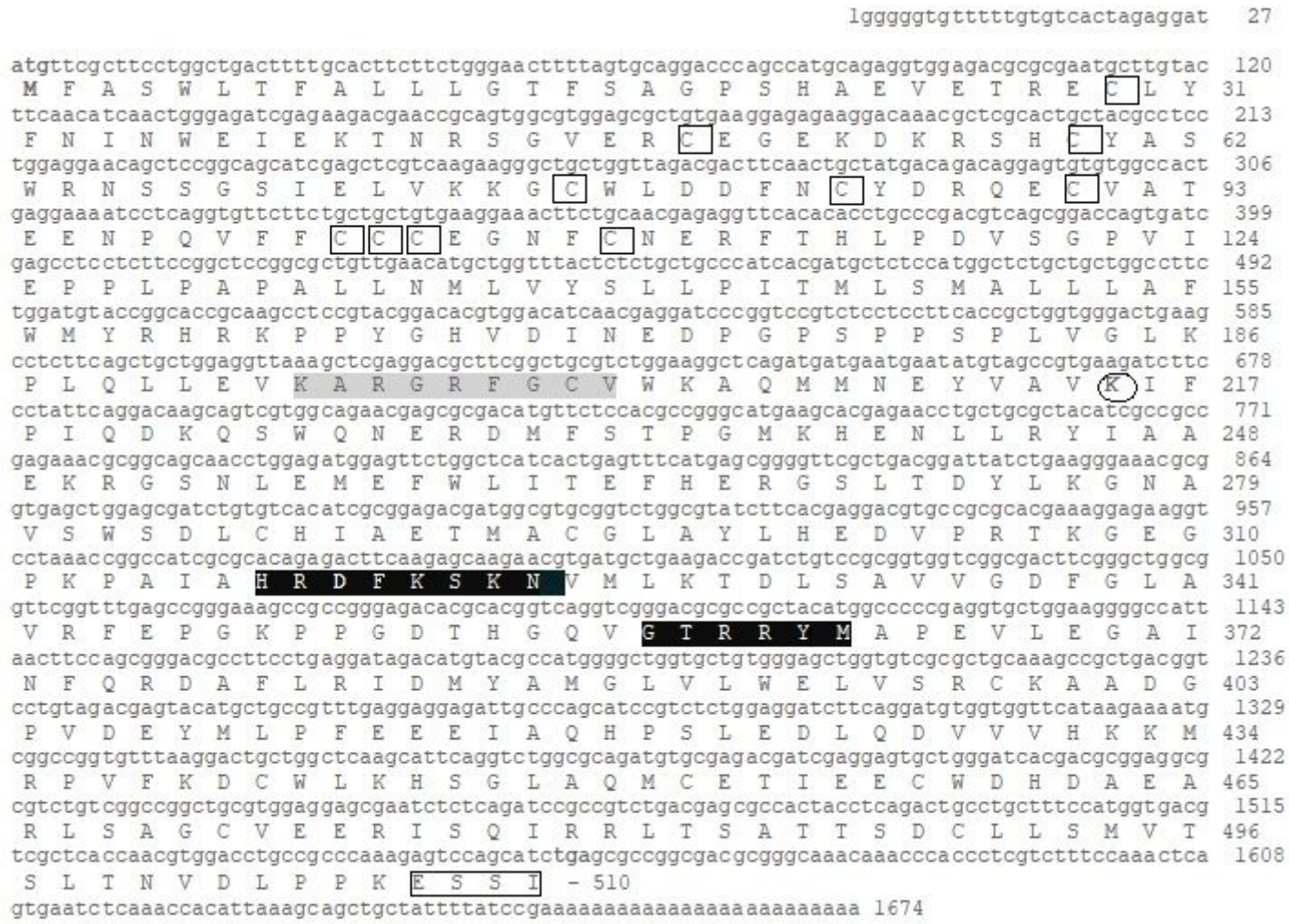

Figure 1

Sequence information of L. rohita ActRIIB. The full-length nucleotide and deduced amino acid sequences of the rohu ActRIIB with numbers are represented on the right side. The start and stop codons are in bold letters. Ten conserved cysteine in extracellular domain are inside rectangular boxes. The two characteristic Ser/Thr kinase motifs are shaded in black. “D319” in bold letters is predicted to be the Ser/Thr kinase active-site. “K215” as the ATP-binding site is shown in circle. The amino acid sequence shaded in grey and underlined is predicted to be the nucleotide binding site. The PDZ-protein binding motif is in the rectangular box.

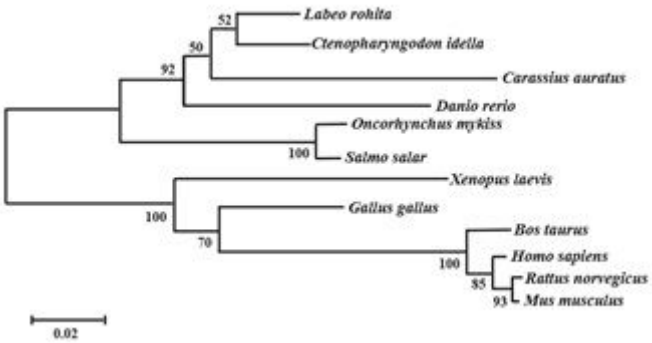

Figure 2

Phylogenetic relationship of L. rohita ActRIIB. The evolutionary relationship with other ActRIIB protein counterparts was analyzed with Mega 6.06 program by bootstrap analysis using neighbor joining with 1000 replicates.

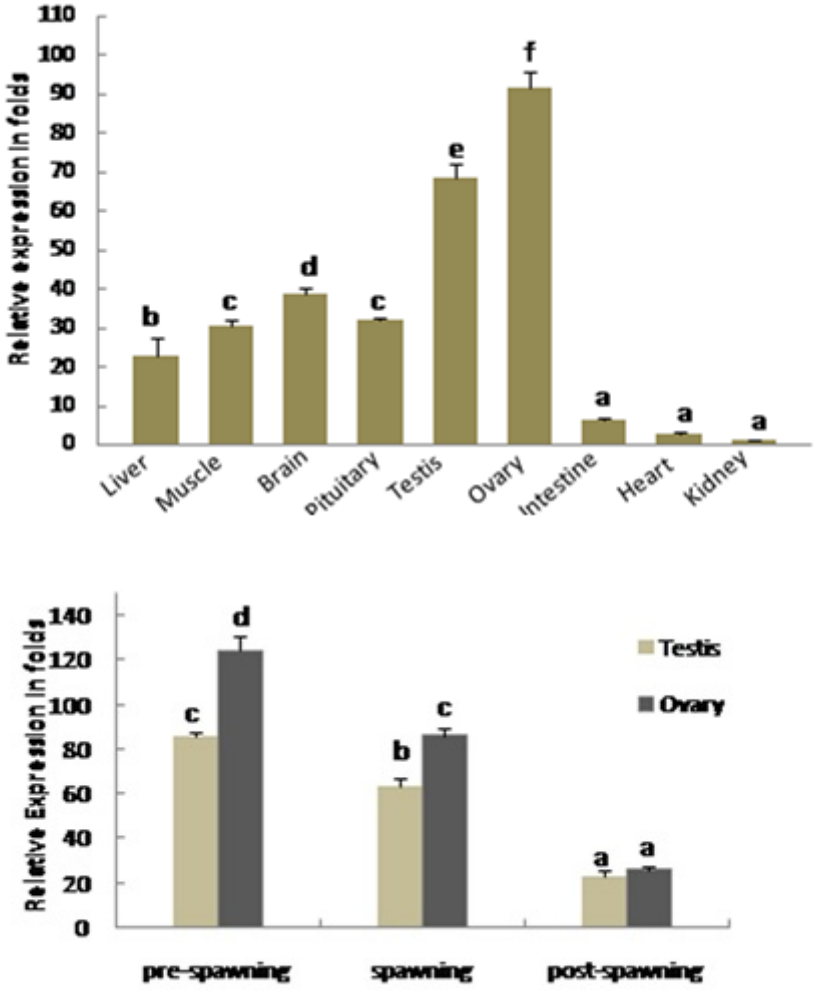

Figure 3

(A) Tissue distribution of ActRIIB transcript in juvenile rohu. Relative expression of ActRIIB mRNA in various tissues of rohu with kidney as the calibrator. Values are mean ± SEM (n=3 for each sample). Bars with different letters show significant differences. (B) Expression analysis of ActRIIB transcript in rohu gonads during different reproductive phases (Pre-spawning, Spawning, Post-spawning). Relative expression is measured in folds with β-actin taken as the reference. Values are mean ± SEM (n=6 for each sample).

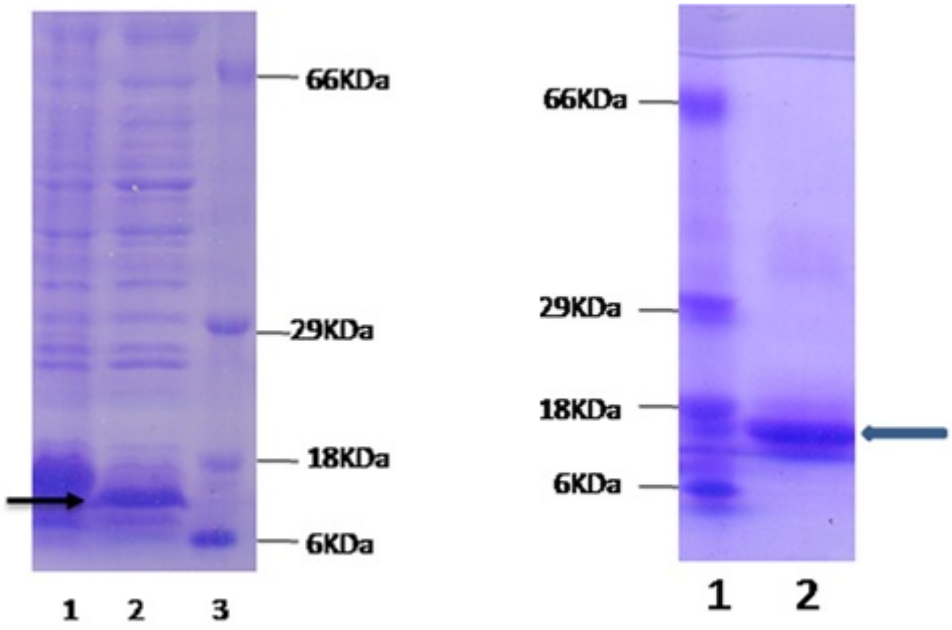

Figure 4

(A) SDS-PAGE (12%) analysis of Rosetta DE3 cells harboring pET28a for rECD-ActRIIB expression. The arrow points towards expressed rECD (~ 14 kDa). (B) Final pooled purified protein in a single band.

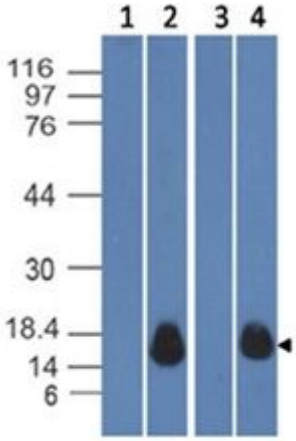

Figure 5

Western blot expression analysis of antibody raised in rabbit against rECD-ActRIIB.

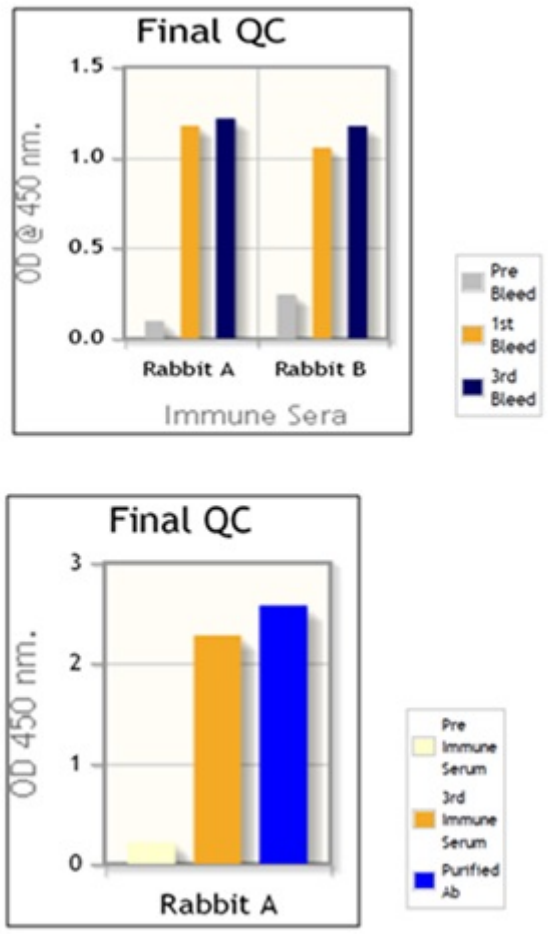

Figure 6

(A) Immune sera of Rabbit A and B from Three bleed stages tested against 200ng of rECD-ActRIIB antigen. (B) Pre and Third Immune sera of Rabbit A along with purified Ab tested against 200ng of recombinant protein.

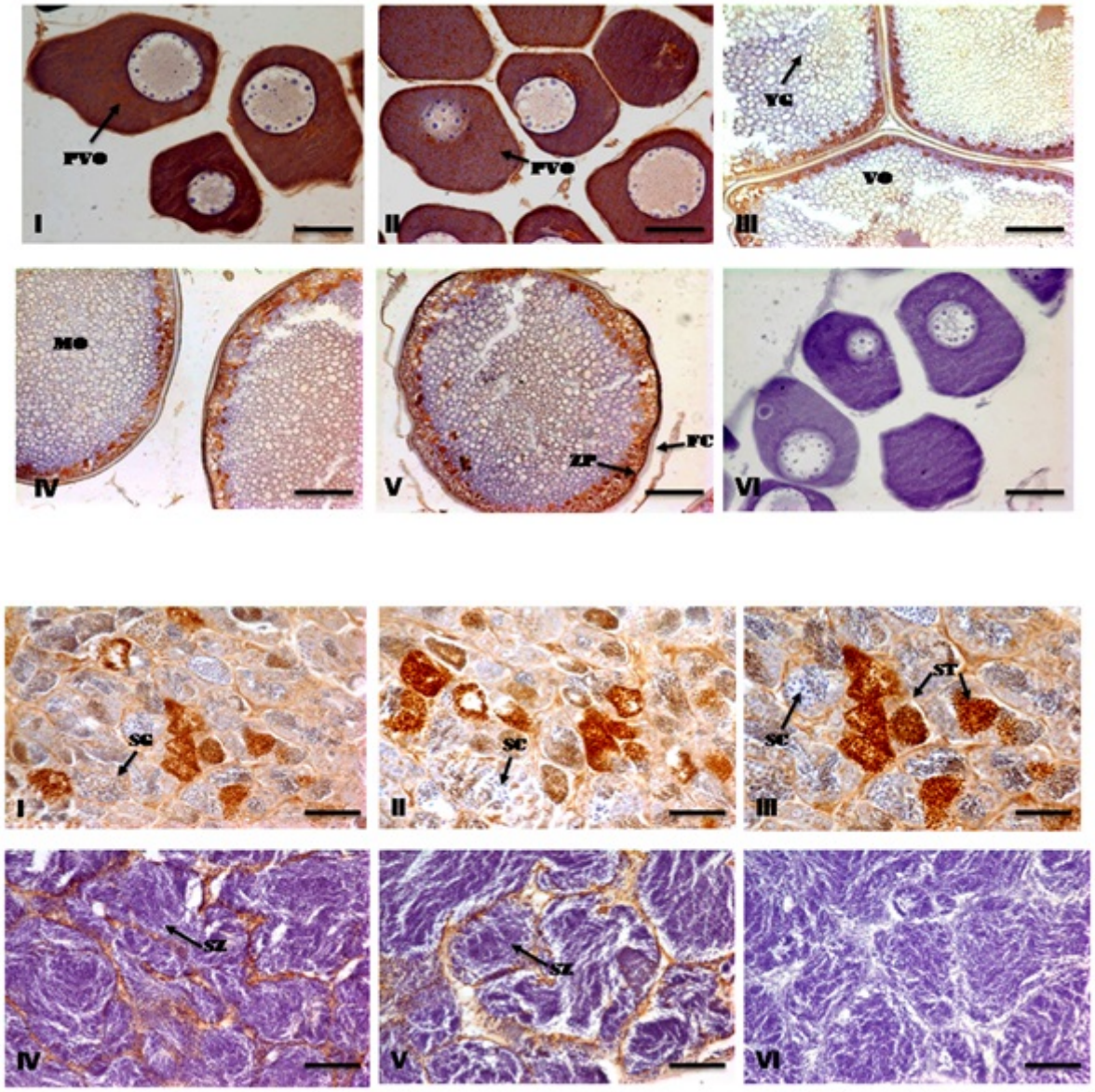

Figure 7

(A) Immunohistochemical staining for ActRIIB in L. rohita ovaries during pre-spawning and spawning stages as shown in ascending order from top left. (i-ii) Pre- vitellogenic stage with primary oocytes (in arrow head) showing strong immunoreactivity to ActRIIB. (iii) Primary oocyte without any immunostain was used as a negative control. (iv) Late vitellogenic stage with both follicular cell layer and oocytoplasm of oocyte vesicles showed intense immunoreactivity of ActRIIB. (v-vi) Mature oocyte with immunostain only in the follicular cell layer and zona pellucida. PVO- Pre-Vitellogenic oocyte, VO- Vitellogenic oocyte, FC- Follicular cell layer, ZP- Zona pellucida, YG- Yolk globules, MO- Mature oocyte. Bar represents: 50 µm (i-iii), 100 µm (iv-vi). (B) Immunohistochemical staining for ActRIIB in L. rohita testis during pre-spawning and spawning stages as shown in ascending order from top left. (i-iii) Spermatogenesis stage with spermatogonia and spermatids showing strong immunoreactivity to ActRIIB. (iv) Immature male rohu in pre-spawning stage without any immunostain was taken as the negative control. (v) Mature male with

spermatozoa showing weak immunoreactivity to ActRIIB. (vi) Mature male rohu in spawning stage taken as negative control. SG-Spermatogonia, SC-Spermatocyte, ST-Spermatids, SZ- Spermatozoa. Bar represents: 50 µm (i-vi).

Supplementary Files

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- SupplementaryTableS1.docx