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Research paper

# Expression profiling of *c-kit* and its impact after esiRNA silencing during gonadal development in catfish

C. Laldinsangi, B. Senthilkumaran*

*Department of Animal Biology, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad 500046, Telangana, India*

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ABSTRACT

Receptor, c-Kit is a member of a family of growth factor receptors that have tyrosine kinase activity, and are involved in the transduction of growth regulatory signals across plasma membrane by activation of its ligand, kitl/scf. The present study analyzed mRNA and protein expression profiles of *c-kit* in the gonads of catfish, *Clarias gariepinus*, using real time PCR, *in situ* hybridization and immunohistochemistry. Tissue distribution analysis revealed higher expression mainly in the catfish gonads. Ontogeny studies showed minimal expression during early developmental stages and highest during 50–75 days post hatch, and the dimorphic expression in gonads decreased gradually till adulthood, which might suggest an important role for this gene around later stages of sex differentiation and gonadal development. Expression of *c-kit* was analyzed at various phases of gonadal cycle in both male and female, which showed minimal expression during the resting phase, and higher expression during the pre-spawning phase in male compared to females. *In vitro* and *in vivo* induction using human chorionic gonadotropin elevated the expression of *c-kit* indicating the regulatory influence of hypothalamo-hypophyseal axis. *In vivo* transient gene silencing using *c-kit*-esiRNA in adult catfish during gonadal recrudescence showed a decrease in *c-kit* expression, which affected the expression levels of germ cell meiotic marker *sycp3*, as well as several factors and steroidogenic enzyme genes that are involved in germ cell development. Decrease in the levels of 11-ketotestosterone and testosterone in serum were also observed after esiRNA silencing. The findings suggests that *c-kit* has an important role in the process of germ cell proliferation, development and maturation during gonadal development and recrudescence in catfish.

## 1. Introduction

Various stages of spermatogenesis that constitute germ cell migration, proliferation, maturation and survival as well as testicular development and function, are under the influence of the tyrosine kinase receptor protein, c-Kit and its ligand, stem cell factor (SCF) in vertebrates (Loveland and Schlatt, 1997; Zhang et al., 2013). The proto-oncogene, *c-kit* is allelic to the *dominant white spotting* (W) locus on chromosome 5 in mouse and belongs to a family of growth factor receptors with intrinsic tyrosine kinase activity that is crucial for transduction of growth regulatory signals across the plasma membrane (Besmer et al., 1993, Yoshinaga et al., 1991; Galli et al., 1993a,b). The KIT receptor comprises of three main functional regions, outer extracellular domain that consists of five immunoglobin-like structures which is essential for ligand binding and dimerization, a transmembrane hydrophobic region that anchors the receptor to the cell membrane and the intracellular kinase domain required for signal transduction conveyed by the specific ligands that results in subsequent autophosphorylation of several tyrosine residues (Sette et al., 2000; Roskoski, 2005). The ligand SCF is expressed in Sertoli cells that lie in close contact with germ cells (Yoshinaga et al., 1991). Apart from its role in reproductive function, c-Kit signalling has been found to be critical for other physiological processes like hematopoiesis, pigmentation, gut movement, as well as nervous system. Further, impairment of the this receptor kinase activity had been found to cause allergies and cancer (Isozaki et al., 1995; Zhang and Fedoroff, 1997; Puxeddu et al., 2003; Alexeev and Yoon, 2006; Edling and Hallberg, 2007; Stankov et al., 2014). In gametogenesis, *c-kit* expression has been shown to be crucial for the development and maintenance of primordial germ cells (PGCs) in both male and female gonads, from embryonic to post-natal stages (Besmer et al., 1993; Nakatsuji and Chuma, 2001; Hutt et al., 2006). In particular, numerous studies have been done in mammals during spermatogenesis that have elucidated a critical role of *c-kit* at all stages of development (Mauduit et al., 1999). Mutations in the loci of c-

*Abbreviations:* dph, days post hatch; esiRNA, endonuclease prepared small interfering RNAs; hCG, human chorionic gonadotropin hormone; IF, immunofluorescence; IHC, immunohistochemistry; ISH, *in situ* hybridization; PEI, polyethylenimine

* Corresponding author.
*E-mail addresses:* bsksl@uohyd.ernet.in, senthilkumaranbalasubramanian@gmail.com (B. Senthilkumaran).

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**Table 1**
List of primers used for cDNA cloning and qPCR analysis.

| Sl. No | Primer name | Primer sequence 5′-3′ | Usage | GenBank Accession number |
|---|---|---|---|---|
| 1. | *c-kit* Dgfw | GGCHCTRATGTCRGARCTRAAGG | RT-PCR | MF093750 |
| 2. | *c-kit* Dgrv | GGCSACYTGGTASGAGAAGC | | |
| 3. | 5P | GCTGAGGAGGTCTTCAGTGTCCAGAGCC | RACE | |
| 4. | 5N | CAGAGTCGGACCTCCAACAGTGCAGGC | | |
| 5. | 3P | GCCTGCACTGTTGGAGGTCCGACTCTG | | |
| 6. | 3N | GGCTCTGGACACTGAAGACCTCCTCAGC | | |
| 7. | 5P#2 | CCTCCCGTTCCCAAAGCACTGGTGCGTC | | |
| 8. | 5N#2 | CTGCAGAGGCCTGCATGCAAGCTTCCC | | |
| 9. | 3P#2 | GCCCGTCTGCCGGTGAAGTGGATGTCT | | |
| 10. | 3N#2 | GCAGTCCTTACCCGGGTGTACCTGTAG | | |
| 11. | 5P#3 | CAGGATGAAGCAAAGGATGGCTGCTGC | | |
| 12. | 5N#3 | GGCTCTCCACCGCTCCCTTCCCAAAGGG | | |
| 13. | 3P#3 | CCCGTCTGCCGGTGAAGTGGATGTCTCC | | |
| 14. | 3N#3 | CATGCGGTCTTGTTGGGAGGCAGACCC | | |
| 15. | *c-kit* RTfw | GCACTGTTGGAGGTCCGACTCTGGTG | qPCR | |
| 16. | *c-kit* RTrv | GGTGCTGGCTCTTACACAGACTGTG | | |
| 17. | 18s rRNA RTfw | GCTACCACATCCAAGGAAGGCAGC | qPCR | AB105163 |
| 18. | 18srRNA RTrv | CGGCTGCTGGCACCAGACTTG | | |
| 19. | *sycp3* RTfw | CCCAGTATAGCCAGAGGCAGAAGCTG | qPCR | KY553233 |
| 20. | *sycp3* RTrv | GAACATGGAGGAGATGGAGAAGAGTC | | |
| 21. | *vasa* RTfw | GGTCTGAGTAAAGTTCGTTATCTG | qPCR | GU562470 |
| 22. | *vasa* RTrv | GCCAACCTTTGAATATCCTCTG | | |
| 23. | *dmrt1* RTfw | ATGGCCGCTCAGGTGGCTCTGCGG | qPCR | FJ596554 |
| 24. | *dmrt1* RTrv | GCGGCTCCCAGAGGCAGCAGGAGA | | |
| 25. | *sox3* RTfw | CACGGTATGAGTAGCCCACCA | qPCR | HQ680982 |
| 26. | *sox3* RTrv | GCGATGGCAGGTGGTGGTGAG | | |
| 27. | *sox9* RTfw | TCTGGCGGCTGCTGAATGAAGG | qPCR | HM149258 |
| 28. | *sox9* RTrv | CTCGGTATCCTCGGTTTCACC | | |
| 29. | *wt1* RTfw | ACGCGCACAGGGTGTTCGA | qPCR | JF510005 |
| 30. | *wt1* RTrv | GGTACGGTTTCTCTCCTTGTG | | |
| 31. | 3β-hsd RTfw | GAGGTAAATGTGAAAGGTACCAA | qPCR | HQ680983 |
| 32. | 3β-hsd RTrv | TAGTACACAGTGTCCTCATGG | | |
| 33. | gata4 RTfw | CAGAGACGACTCTCTGCTTCCAG | qPCR | KT031389 |
| 34. | gata4 RTrv | CCCCGTGGAGCTTCATGTAGAG | | |
| 35. | *11β-h* RTfw | GGCAGTGGAGCGAATGCTGAA | qPCR | HQ680986 |
| 36. | *11β-h* RTrv | GCACCCCGGGGAACCTTGAGC | | |
| 37. | 17β-hsd12 | AGCCATCGAGAGCAAGTACCATGT | qPCR | JN848590 |
| 38. | 17β-hsd12 | AAGCCGAGTCATCTGACAAACCGA | | |
| 39. | Activin | TCGCAGAGCCTGTTGATGCT | qPCR | KF956110 |
| 40. | Activin | GCTGACACTCAGGGTCTGCA | | |

Kit or its ligand SCF have resulted in aberrant PGC development and sterility in mice (De Miguel et al., 2002). The expression of c-Kit receptor was observed mainly in spermatogonia and Leydig cells of adult mouse testis (Manova et al., 1990; Zhang et al., 2013) and it has been detected at post-natal day 5 exclusively in differentiating type A and B spermatogonia as well as in primary spermatocytes (Manova et al., 1993; Sette et al., 2000). A truncated c-Kit receptor without extracellular, transmembrane and part of the intracellular tyrosine kinase domain, has also been detected in spermatids and spermatozoa (Albanesi et al., 1996). One of the key roles of *c-kit* expression is the maintenance of ratio between self-renewing and differentiating spermatogonial stem cells which is normally maintained at 1.0, and any deviations might lead to tumours in the seminiferous epithelium (Bokemeyer et al., 1996; Zhang et al., 2011). In addition, the activation of c-Kit signalling has a decisive role in spermatogenesis by activating meiosis at specific time points, and then it leads to subsequent expression of early meiotic markers such as *Dmc1* and *Sycp3* in mammals (Vincent et al., 1998; Di Carlo et al., 2000).

Although the function and expression of *c-kit* has been well studied in mammalian systems, only few reports have been documented on its role in fish reproduction. Reproductive physiology in lower vertebrates such as fish shows strategic continuous mature gonads after initial development as well as seasonal pattern, occasionally different between sexes. Hence, using fish as a model may provide novel information on c-kit's role in gonads undergoing reproductive cycle. Earlier studies in model organisms such as zebrafish, *Danio rerio*, were restricted mostly pertaining to the processes such as haematopoiesis, melanogenesis and oogenesis (Ransom et al., 1996; Weinstein et al., 1996; Parichy et al., 1999, Rawls and Johnson, 2000; Yao and Ge, 2010, 2013). Receptor c-Kit has been identified as a male germ cell marker in spermatogonial cells of the Japanese medaka, *Oryzias latipes*, dogfish *Scyliorhinus canicula* and rohu *Labeo rohita* (Hong et al., 2004; Loppion et al., 2008; Bosseboeuf et al., 2013) and there has been no detailed study on the involvement of this gene in relation to germ cell development, proliferation and maturation especially in annual breeders such as catfish. Annual breeding teleosts show distinguished pattern of germ cell maturation and gonadal recrudescence during first and cyclic stages of gametogenesis, thereafter during seasonal reproductive cycle.

Present study aims to analyze the role of *c-kit* during various stages of germ cell development in particular spermatogenesis, using the African air-breathing catfish, *Clarias gariepinus* as an animal model. Initially, cDNA of *c-kit* was cloned from catfish testis, which was followed by real-time PCR (qPCR) analysis of expression in various tissues, during gonadal ontogeny and seasonal reproductive phases. The response of this gene to gonadotropins using human chorionic gonadotropin (hCG) was analyzed, *in vitro* and *in vivo*. Localization of *c-kit* mRNA and protein in the testis was done using *in situ* hybridization (ISH), immunohistochemistry (IHC)/Immunofluorescence (IF), respectively. Further, *in vitro* and *in vivo* transient gene silencing using *c-kit*-esiRNA by polyethylenimine (PEI) mediated transfection (Höbel and Aigner, 2010, 2013) was performed in adult catfish during gonadal recrudescence to analyze the effect of *c-kit* on various germ cell markers, other factors and steroidogenic enzyme genes related to germ cell maturation and spermatogenesis in catfish.

## 2. Materials and methods

### 2.1. Catfish breeding and sample collection

Adult catfish, *C. gariepinus*, reared in fresh water tanks (25 ± 2 °C) under ambient photothermic conditions, were used for this study. Catfish undergo a seasonal pattern of reproductive cycle annually, divided in four different phases, preparatory (February–April), pre-spawning (May–June), spawning (July–November) and regressing or post spawning phase (December–January). This can be a useful characteristic in analysing the roles of genes during gonadal development as well as recrudescence.

*In vitro* fertilization, rearing and maintenance of catfish were done as per the procedure described by Raghuveer et al. (2011). Sampling of fishes was performed by following the general guidelines and with the approval of the Institutional Animal Ethics Committee, University of Hyderabad (CPCSEA, Inst. Reg. No.151/1999 dt. 22.07.1999).

### 2.2. Molecular cloning of c-kit from catfish testis

The molecular cloning of *c-kit* was done as per the method described by Laldinsangi and Senthilkumaran (2018). In brief, total RNA was prepared from adult catfish testis which was then used to prepare first strand cDNA using Primescript RT-PCR (Takara Bio Inc., Japan) as per the manufacturer's protocol. Degenerate primers were used for PCR amplification and are listed in Table 1. The amplicon was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), then sequenced bidirectionally, analyzed and confirmed as catfish *c-kit* partial cDNA through BLAST. Gene specific primers (GSP) were designed using the nucleotide sequence of partial cDNA of *c-kit* to obtain the full length sequence by Rapid Amplification of cDNA Ends (RACE). Primers used for touchdown PCR are also listed in Table 1. RACE products were cloned into pGEM®-T easy vector (Promega), subsequently sequenced bidirectionally and analyzed by NCBI-BLAST.

### 2.3. Quantitative RT-PCR (qPCR)

Gene expression analysis was determined using qPCRwith SYBR green detection method. The specific primers used for all the experiments (Table 1) were designed for the amplicon length of ~200 bp. Caution was taken to exclude genomic DNA by designing at least one primer to span an exon-exon boundary. The reactions were set up in triplicates in MicroAmp® 96-Well pates with Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in an ABI Prism® 7500 fast thermal cycler (Applied Biosystems) according to the manufacturer's universal thermal cycling conditions. Melting-curve analysis was done to check the specificity of the PCR amplification and no-template control did not display any amplification. Cycle threshold (Ct) values from the exponential phase of PCR amplification were used for generating ΔCt value with *18S rRNA* taken for the normalization against the expression of target gene. Relative expression of the genes were calculated using $2^{-\Delta Ct}$ method.

### 2.4. Tissue distribution, reproductive cycle and ontogeny studies

Adult catfish (n = 5), both male and female from late preparatory phase were sacrificed and different tissues (brain, heart, muscle, liver, kidney, testis, ovary) were then dissected out. Testis samples were collected for phase expression analysis at different time intervals of the reproductive cycle. Embryo samples were also collected after IVF at different hours post fertilization. In catfish, gonads differentiate and morphologically distinguishable at ~35–50 days post hatch (dph), into either testis or ovary. Samples of developing gonads from both male and female were then collected at different stages of development (0, 10, 20, 40, 50, 75, 100, 200 dph) as well as adults. Mesonephric-gonadal complex was collected from 0 to 40 dph fingerlings, and gonads were collected from 50 dph onwards. Wherever required, sample pooling was done to obtain five biological samples that were used for the experiments. All samples were stored briefly at −80 °C. Total RNA extraction, cDNA synthesis and qPCR (performed in triplicates for each sample) were done as described earlier.

### 2.5. hCG induction in vitro and in vivo

Gonadotropin induction, *in vitro* and *in vivo* using hCG at late preparatory phase on adult catfish were performed as per the procedure described by Murugananthkumar et al. (2017). In brief, for *in vitro* induction, testis was dissected from an adult male during preparatory phase under sterile conditions and kept it in ice-cold Leibovitz (L-15) culture medium (Sigma). Using a McIIwain tissue chopper (Vibratome, Ted Pella Inc., Redding, CA, USA), testicular slices of about 50 µm thickness were cultured in L-15 medium supplemented with 10 mM HEPES and antibiotics (penicillin, 100 IU/ml; streptomycin, 0.1 mg/ml). The slices were maintained for 24 h at 20–22 °C with 100 IU/ml of hCG (Trade name: Pubergen; Sanzyme Ltd., Shameerpet TS, India) added to the medium. Fish physiological saline (0.6% NaCl w/v) was used for control. Testis samples were then collected from both hCG treated and control at different time points 2, 4, 6, 12, 20, and 24 h. Total RNA extraction and cDNA synthesis were done and relative expression was calculated as previously explained.

For *in vivo* induction (long term effect), an osmotic pump (ALZET® osmotic pumps, Cupertino, 116 CA, USA) filled with 5000 IU of hCG in 100 µl of saline was implanted into the peritoneal cavity, close to the gonads, through a small incision of about 8 mm that was cut in the intraperitoneal region. Physiological saline was used for control groups. Fishes were anesthetized with 100 mg/L of MS-222 (Sigma). The incision was carefully sutured using sterile 30 mm catgut and the fishes were monitored in glass tanks for 3 days Samples were taken after 21 days.

### 2.6. Western blot analysis

Western blot analysis was carried out using C-KIT polyclonal antibodies (Cat. No. LS-C160001; Life Span Biosciences, Seattle, WA, USA) raised against the conserved C-terminal regions of human C-KIT which showed ~93% homology with the conserved region of catfish. The homologous amino acid residue region of C-KIT for human and catfish are KICDFGLARDITTDSNYVVKGNARLPVKW and KICDFGLARDIKNDSNYVVKGNARLPVKW, respectively. Tissue lysate was extracted from adult catfish testis by homogenizing with ice cold 1 M Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, and ProteoBlock™ protease inhibitor cocktail (Thermo Scientific). After centrifugation at 15,000 × *g* at 4 °C, supernatant was pipetted out and protein concentration was estimated using Bradford method. The homogenate containing a protein concentration of 100 µg was run on a 12% SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane (Pall Life sciences, Port Washington, NY, USA). The membrane was blocked with 5% skimmed milk in Tris-buffered saline (TBS) for 1 h at RT. Washing was done thoroughly in TBS with 0.1% Tween 20 (TBST), and then incubated with a 1: 5000 dilution of polyclonal anti-C-KIT antibody overnight at 4 °C. The membrane was washed and incubated with alkaline phosphatase conjugated goat anti-rabbit IgG secondary antibody (Merck Bangalore Genei, Bengaluru, India) for 1 h at RT. Protein bands were visualised by BCIP-NBT substrate-catalysed detection (Roche, Roche Diagnostics GmbH, Mannheim, Germany).

### 2.7. ISH, IHC and IF

ISH was performed in adult catfish testis in view of localizing mRNA transcripts as per the method described earlier (Laldinsangi and Senthilkumaran, 2018). In brief, testis dissected from adult catfish were fixed using 4% paraformaldehyde (PFA), washed and immersed in OCT

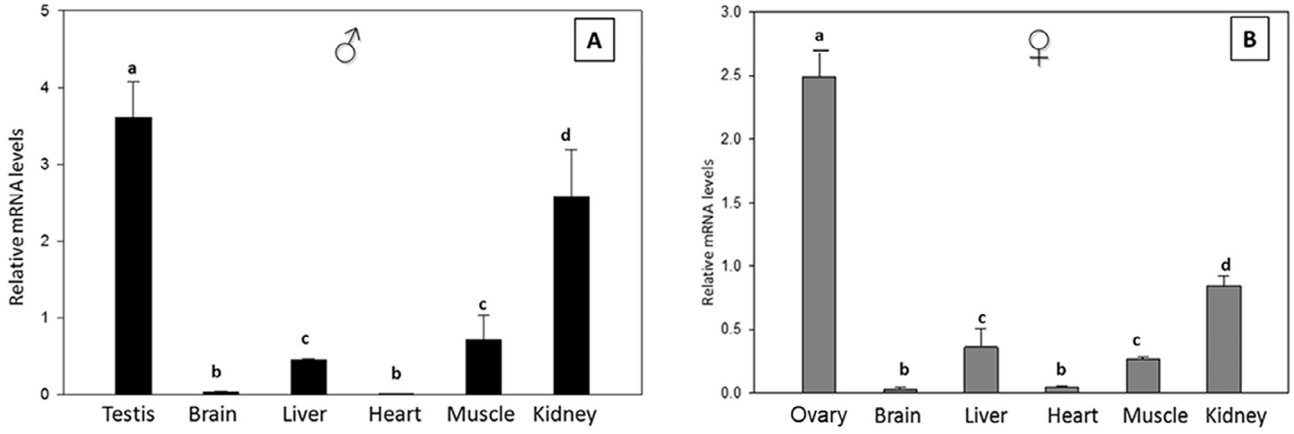


**Fig. 1.** qPCR analysis of *c-kit* expression in various tissues of catfish in male (A) and female (B). The *c-kit* expression levels varied in different tissues, but showed significantly ($P < 0.05$) higher expression in both male and female gonads, moderate expression in kidney and lower in liver and muscle. The relative expression of *c-kit* was normalized with *18S* rRNA and the values were calculated using $2^{-\Delta ct}$ method. Data (n = 5) were expressed as mean ± SEM. Mean with different letters differs significantly while mean with same letters are not significant ($P < 0.05$; ANOVA followed by Student-Newman-Keuls' post hoc test).

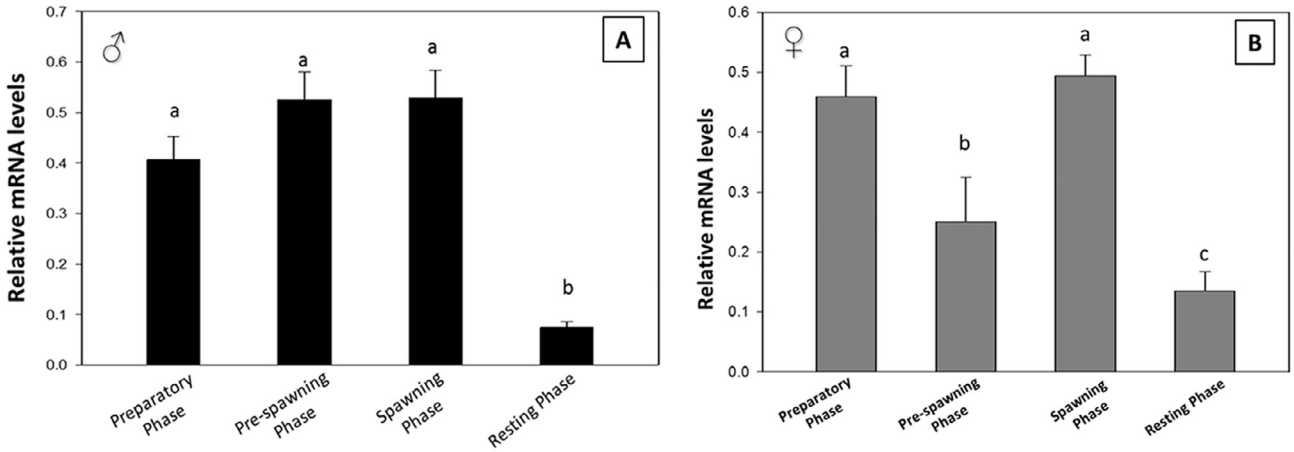


**Fig. 2.** qPCR analysis of *c-kit* expression during different phases of the reproductive cycle in catfish, *Clarias gariepinus* in male (A) and female (B). Expression levels of *c-kit* in the different phases of gonadal cycle were low during resting phase for both male and female, while males showed significantly elevated levels during the preparatory to spawning phases (A) compared to significantly low expression during pre-spawning phase in females (B). Other details are as in the Fig. 1.

compound (Leia, Buffalo Grove, IL, USA) and frozen for about 2–4 h. Tissue sections of 7 µm were cut using cryostat (Leica CM1850). Digoxigenin (DIG) RNA labelling mix (Roche) was used to synthesize sense and antisense 'cRNA' probes as per the manufacturer's instructions. The probes were then added to the slides and incubated at 50 °C overnight in a sterile RNase free incubator after which DIG-ALP antibody diluted 1:1000 in maleic acid buffer (Roche) were added to the slides and kept at 4 °C overnight. This was followed by addition of DIG washing buffer (Roche), BCIP-NBT (Roche) and nuclear red as a counter stain. Images were obtained using Leica DM6 B digital research microscope (Leica Microsystems Gmbh, Wetzlar, Germany).

IHC was performed in adult catfish testis sections in order to localize c-kit using the anti-C-KIT antibody (Cat. No. LS-C160001, Life Span Biosciences), as per the method described by Laldinsangi and Senthilkumaran (2018). In brief, adult catfish testis was fixed in Bouin's fixative (saturated picric acid: formaldehyde: glacial acetic acid, 15:5:1) and embedded in paraplast (Sigma) post dehydration. Tissue sections of 6 µm thickness were fixed on to Poly-L-Lysine coated glass slides and deparffinized, followed by rehydration and endogenous peroxidase blocking. After sufficient treatment, anti-C-KIT polyclonal antibody (1:1000) or pre-adsorbed antibody with excess antigen of C-KIT peptide (for negative control) were then added onto the sections, and incubated in a humid chamber at 4 °C overnight. The slides were then washed adequately before adding secondary antibody, followed by development with 3′3′-diaminobenzidine (DAB) as chromogen and $H_2O_2$ (Vector Laboratories) as a substrate. The slides were mounted after sufficient colour development. Images were obtained using Q-capture Pro 6 software (Quantitative Imaging Corporation) with Micropublisher 3.3 RTV-CCD camera in CX-41 Olympus Microscope (Olympus Corporation, Tokyo, Japan).

For IF, testis fixation and sectioning was done in freezing medium as described by Murugananthkumar and Senthilkumaran (2016). After blocking with normal goat serum and then incubating with primary antibody C-KIT overnight, the sections were incubated with FITC-conjugated anti-rabbit secondary antibody. Pre-adsorbed antibody was used for negative control. The IF images were taken with an IX81 Olympus Microscope (Olympus Corporation), containing Cell Sens dimension software.

### 2.8. Catfish primary testicular culture

Primary testicular cell culture of catfish was prepared as per the protocol of Murugananthkumar and Senthilkumaran (2016). In brief, adult male catfish was anesthetized using 100 mg/dl MS222 (Sigma) and disinfected with 70% ethanol. Dissected testis was washed several times using sterile PBS and sliced carefully in Leibovitz (L-15) medium, washed, incubated with 0.25% collagenase type I and 0.005% DNase for 15 min at RT. The cells were gently dispersed with the blunt end of a

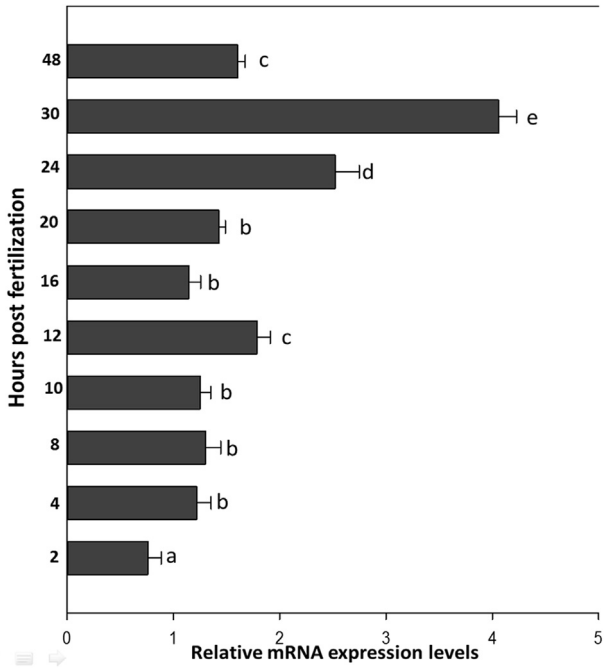


**Fig. 3.** qPCR analysis of *c-kit* expression during various stages of embryological development in catfish. Embryonic stages post fertilization show a gradual and constant expression of *c-kit* until 20 h and a significant ($P < 0.05$) increase was observed after 24 h at the hatched larva stage. Other details are as in the Fig. 1.

syringe, filtered with a 40 µm cell strainer and centrifuged at 100*g* at 4 °C. The pellet was re-suspended in Dulbecco's modified Eagle's medium (DMEM) and ~1 X $10^6$ cells were plated into several wells of Corning® Costar® 24 well cell culture plates (Sigma) and a freshly prepared medium consisting of DMEM, 10% FBS, 1X Glutamax and antibiotic and antimycotic was then added and incubated at 30 °C supplied with 5% $CO_2$. Cell viability was assessed using trypan blue staining. The culture was incubated for 24 h prior to use in transfection experiments.

### 2.9. In vitro esiRNA-PEI transfection

Using the ~500 bp cDNA of *c-kit* cloned from catfish, endonuclease prepared small interfering RNA (esiRNA) was commercially synthesized (Sigma). Before transfection, the culture medium was carefully removed from the plates and 100 µl of OPTI-MEM® I solution was added. Control siRNA (MISSION siRNA Fluorescent Universal Negative Control #1, Cat. No. SIC007 conjugated with 6-FAM, Sigma, 10 ng/µl) was used for control groups. In addition, to rule out any off target effects, PEI alone as well as *c-kit*-esiRNA with no PEI was also added in separate wells as additional controls. For transfection, control siRNA (control group) and varying concentrations of *c-kit*-esiRNA (10, 20, 40 and 100 ng/µl) were complexed with branched PEI (MW 25 K, Sigma) in sterile HEPES-NaCl buffer, pH 7.4 by incubation for 20 min at RT which were then added to the respective wells followed by 12 h incubation. The OPTI-MEM®I and control siRNA/esiRNA-PEI mixture were then discarded after the transfection and replaced with cell culture medium as stated before. The cells were incubated for 24 h post transfection and then collected for further analysis. Total RNA was prepared from the collected samples, and relative expression of certain testis related genes post transfection were analyzed and calculated with qPCR as explained before. Transfection efficiency was analyzed by transfecting the cell culture with control siRNA and microphotographed in an IX81 Olympus Microscope (Olympus Corporation) with Cell Sens dimension software.

### 2.10. In vivo esiRNA-PEI transfection

For *in vivo* transfection, adult (~2 year old) catfish (n = 5) undergoing prespawning phase were used for the experiment. Branched PEI (Sigma) was dissolved in sterile HEPES-NaCl pH 7.4, and combined with 3 µg of *c-kit*-esiRNA as per the protocol described by Höbel and Aigner (2010, 2013) and kept at RT for 30 min to form an esiRNA-PEI complex for transfection (Falco et al., 2009; Zhou et al., 2012). The fishes were anesthetized with 100 mg/L of MS-222 (Sigma) and its ventral area was sterilized with ethanol. To expose the testis, a small incision of about 10 mm was cut above the pelvic fin junction with a sterile fine scalpel. The esiRNA-PEI complex was then directly injected into the testis base lobe with a sterile 1 ml microsyringe. Control siRNA-PEI complex or PEI alone was injected for control groups. After injection the incision was then sutured with sterile 30 mm catgut and treated with antibiotics. The fishes were monitored and maintained separately for 3 days in individual tanks, and tissue samples were taken at different

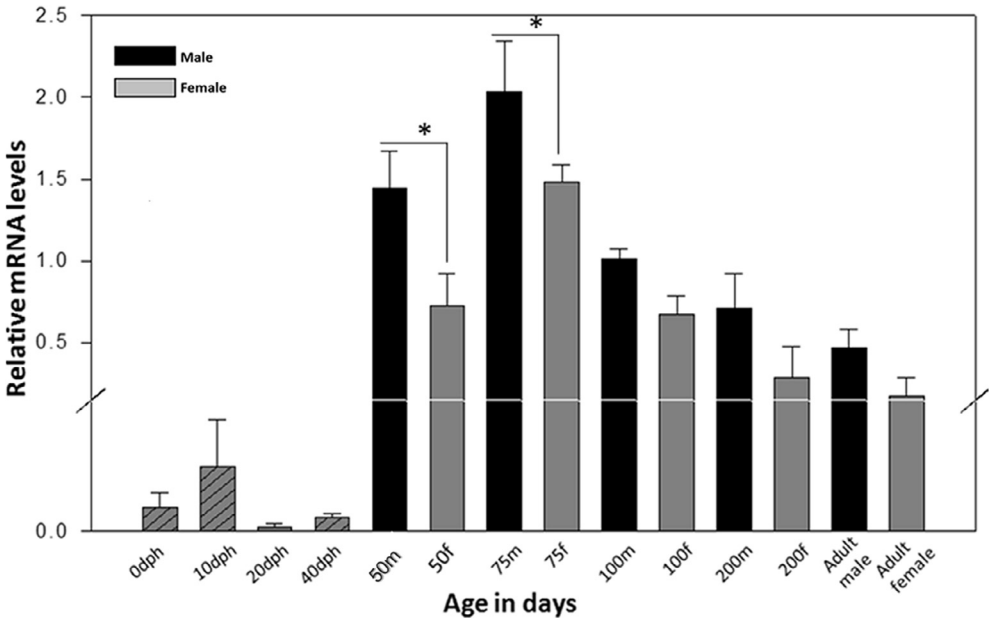


**Fig. 4.** qPCR analysis of *c-kit* expression during various stages of development of catfish. Levels of *c-kit* expression were low during early stages of development and peaked during 50–75 dph, where a significantly higher expression was observed in males during 50 and 75 dph. Data (n = 5) were expressed as mean ± SEM. (*, $P < 0.05$; ANOVA followed by Student-Newman-Keuls' post hoc test).

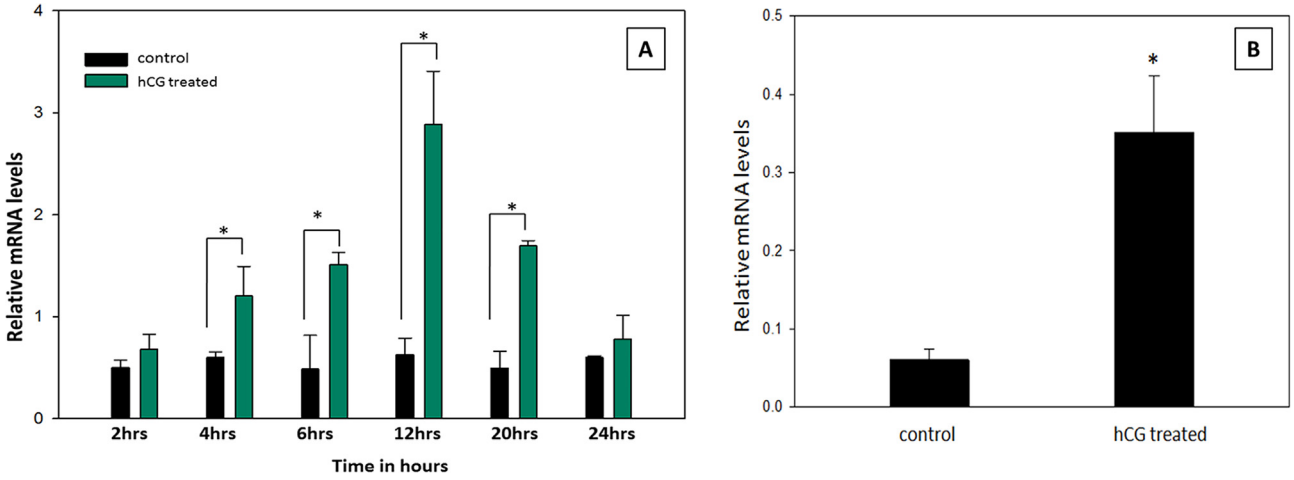


**Fig. 5.** qPCR analysis of *c-kit* mRNA expression levels after treatment with hCG *in vitro* in testicular slices (A) and *in vivo* (B) at different time intervals. *In vitro* induction using hCG increased *c-kit* expression significantly ($P < 0.05$) from 4 h onwards when compared to the control sample (A). There was a also significant increase in *c-kit* expression after the period of *in vivo* induction (21 days), when compared to control (B). Other details are as in the Fig. 4.

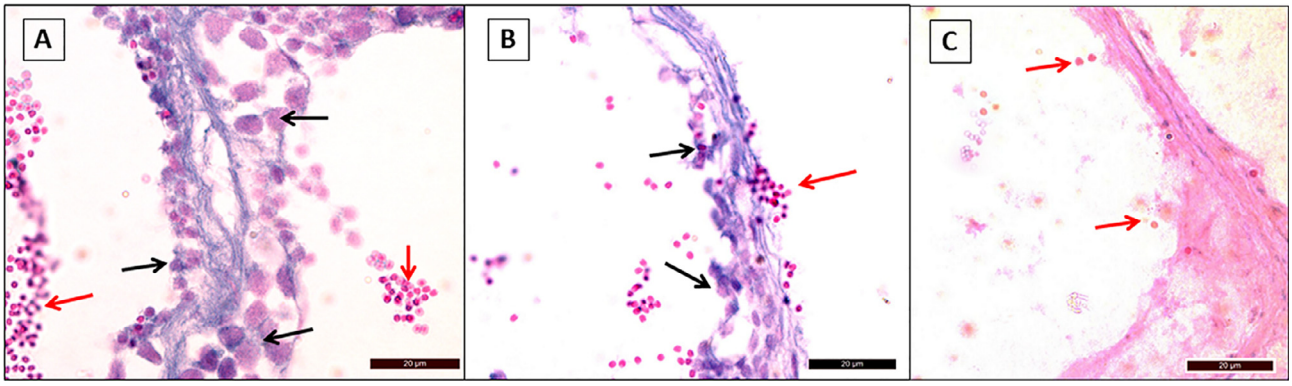


**Fig. 6.** *In situ* hybridization of *c-kit* mRNA in adult testis of catfish. Testis of adult catfish displayed positive signals in spermatocytes and spermatogonia indicated by black arrows (A and B). Signals were not detected in mature sperm/spermatids (red arrows). Sense probe of *c-kit* did not show any signal (C). Counterstain was done with nuclear red. Scale bars indicate 20 μm for all images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

intervals by sacrificing the fishes at approximately 48 and 72 h post transfection which were snap frozen using liquid nitrogen and stored briefly in −80 °C for further analysis.

### 2.11. Estimation of serum 11-ketotestosterone (11-KT) and testosterone (T) levels post c-kit-esiRNA-PEI and control siRNA transfection in catfish

Serum samples were obtained after 3 days post transfection of control or *c-kit* esiRNA. Disposable syringes were used to draw blood from dorsal vein of catfish (n = 5) and was centrifuged at 1000 × *g* for 10 min at 4 °C to separate serum. The serum was then pipetted out carefully and then stored briefly at −80 °C for further analysis. Levels of 11-KT and T in catfish serum were estimated after transfection by enzyme immunoassay (EIA) kit (Cayman, Ann Arbor, MI, USA) as per the manufacturer's protocol. The sensitivity of Cayman EIA kits for 11-KT and T measurement is 1.3 and 6 pg/ml, respectively and the assay validation was done as per the protocol described by Swapna et al. (2006). Assays were done in triplicates for each independent sample (n = 5) and the serum sample dilutions were in parallel with the standard dilutions.

### 2.12. Statistical analysis

All the data were expressed as mean ± standard error of mean (SEM). Significance among groups was tested by Analysis of variance (ANOVA) followed by Student's–Newman–Keuls' test using SigmaPlot 12.0 software (Systat Software Inc., Chicago, IL, USA) for most of the analysis. For the esiRNA transfection results, Mann–Whitney test one way ANOVA on ranks was done, followed by Student's–Newman–Keuls' post hoc test. Differences among groups were considered significant at $P < 0.05$.

## 3. Results

### 3.1. Molecular cloning of c-kit from testis of catfish

Initially, partial cDNA fragment of 372 bp of *c-kit* was cloned from testis of juvenile catfish using degenerate primers' RT-PCR. The cDNA fragments of 5′ and 3′ ends were then acquired by RACE to obtain almost the full length cDNA of 1.5 kb that encodes a putative protein of 530 amino acids which contains signature conserved tyrosine kinase domains to designate *c-kit*, which was adequate enough to perform expression analysis required for this study. Further, the almost full length cDNA with deduced amino acid sequence was confirmed as *c-kit* homologue through BLAST. The nucleotide and deduced amino acid sequences have been submitted to GenBank and the accession number is MF093750. Multiple sequence alignment of deduced amino acid sequences (Supplementary Fig. 1) was done based on the *c-kit* sequences of several teleost species using the ClustalO alignment software from European Bioinformatics Institute website (http://www.ebi.ac.uk/

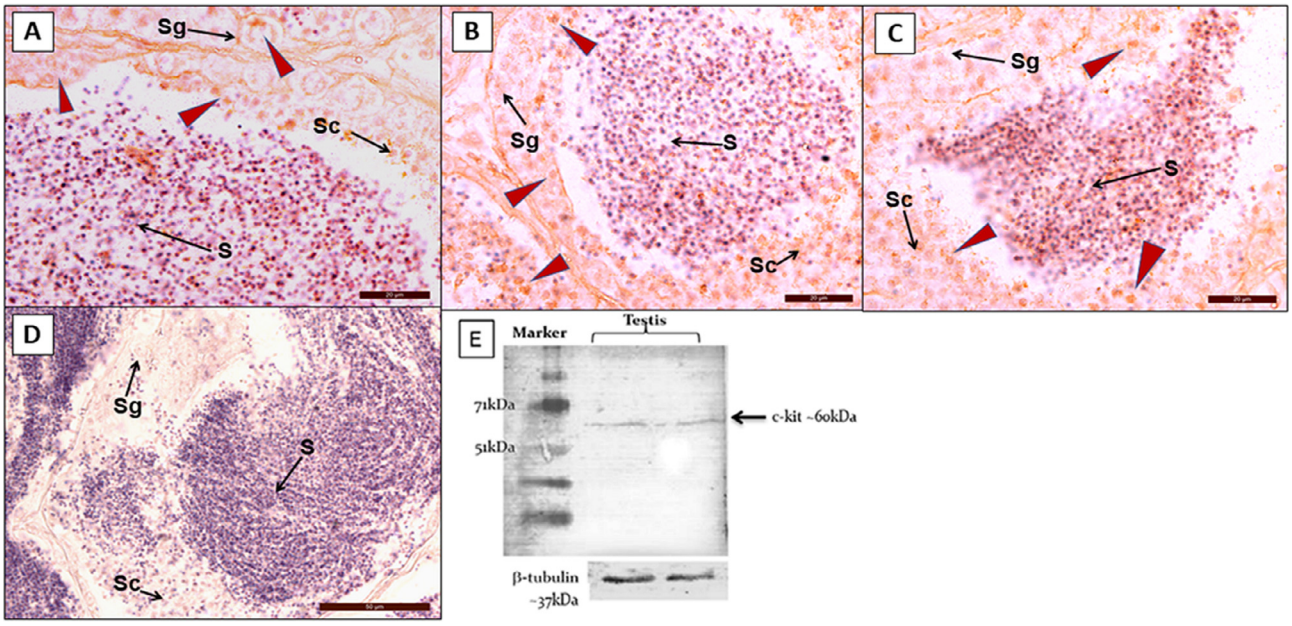


**Fig. 7.** Localization of c-kit protein in catfish testis using immunohistochemical staining. Immunoreactivity of c-kit was observed in spermatogonia and spermatocytes (A–C, indicated by red-arrowheads), while negative control with pre-adsorbed antibody with excess c-kit antigen (D) did not show any positive signal. Abbreviations: Sg – Spermatogonia, Sc – Spermatocytes, S – Sperm/spermatids. Scale bars indicate, A and C: 20 μm, B and D: 50 μm. Counter-stained with hematoxylin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

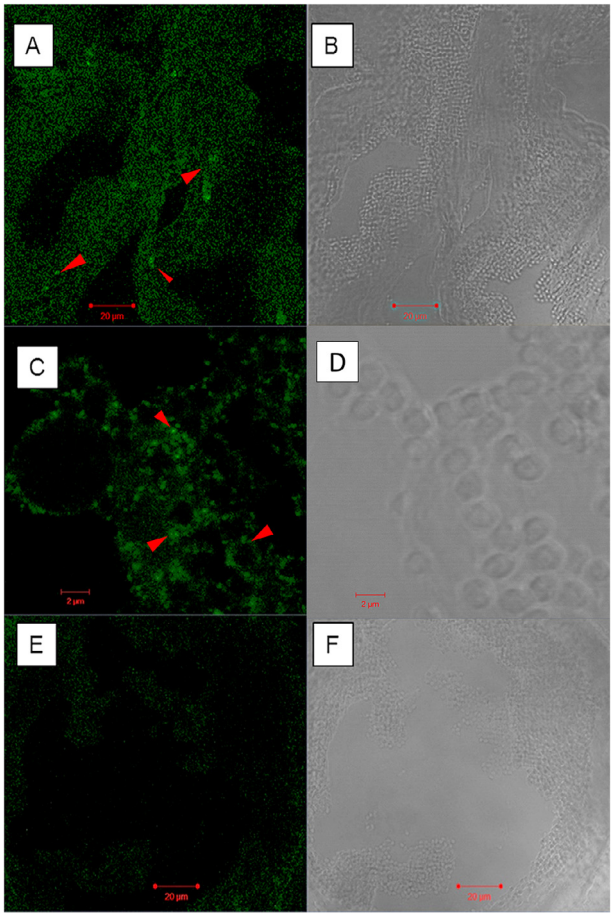


**Fig. 8.** Localization of c-kit protein in catfish testis using immunofluorescence stained with FITC. Positive signals were observed in spermatogonia and spermatocytes (A and C) compared to negative control (E) which corroborates the results obtained from IHC and ISH. B, D and F represent the corresponding phase contrast images. Scale bars indicate, A, B, E, and F: 20 μm; C and D: 2 μm.

Tools/msa/clustalo/).

### 3.2. Tissue distribution, reproductive cycle and ontogeny expression analysis of c-kit in catfish

The expression levels of *c-kit* revealed variability in different tissues, but showed significantly ($P < 0.05$) higher expression in gonads of both male and female, and moderate expression in kidney and lower in liver and muscle (Fig. 1A and B). Expression of *c-kit* during various phases of gonadal cycle in both male and female showed minimal expression during resting phase, with significantly elevated levels during the preparatory to spawning phases in males (Fig. 2A), while this expression levels are low during pre-spawning phase in females when compared to males (Fig. 2B). The *c-kit* expression during embryonic stages post fertilization showed a gradual and constant expression until 20 h and expression significantly ($P < 0.05$) increased after 24 h at the hatched larva stage (Fig. 3). Ontogeny studies showed minimal expression during early stages of development and highest during 50–75 dph, where males showed a significantly higher expression during 50 and 75 dph (Fig. 4). The dimorphic expression decreased gradually by adulthood. The high expression of *c-kit* during this stage is comparable to preparatory phase results where gonadal recrudescence is evident in annually reproducing teleosts.

### 3.3. hCG induction in vitro and in vivo

Induction using hCG, both *in vitro* and *in vivo*, showed a positive effect on the expression levels of *c-kit*. *In vitro* induction of catfish testis using hCG indicated a significant ($P < 0.05$) elevation of *c-kit* expression from 4 h onwards when compared to the control samples (Fig. 5A). In the *in vivo* induction, there was a significant increase in *c-kit* expression after the period of induction (21 days), when compared to control (Fig. 5B).

### 3.4. ISH and immunolocalization of c-kit in catfish testis

ISH was performed to confirm the specific localization of *c-kit* mRNA transcripts in the adult catfish testis sections. The spermatogonia

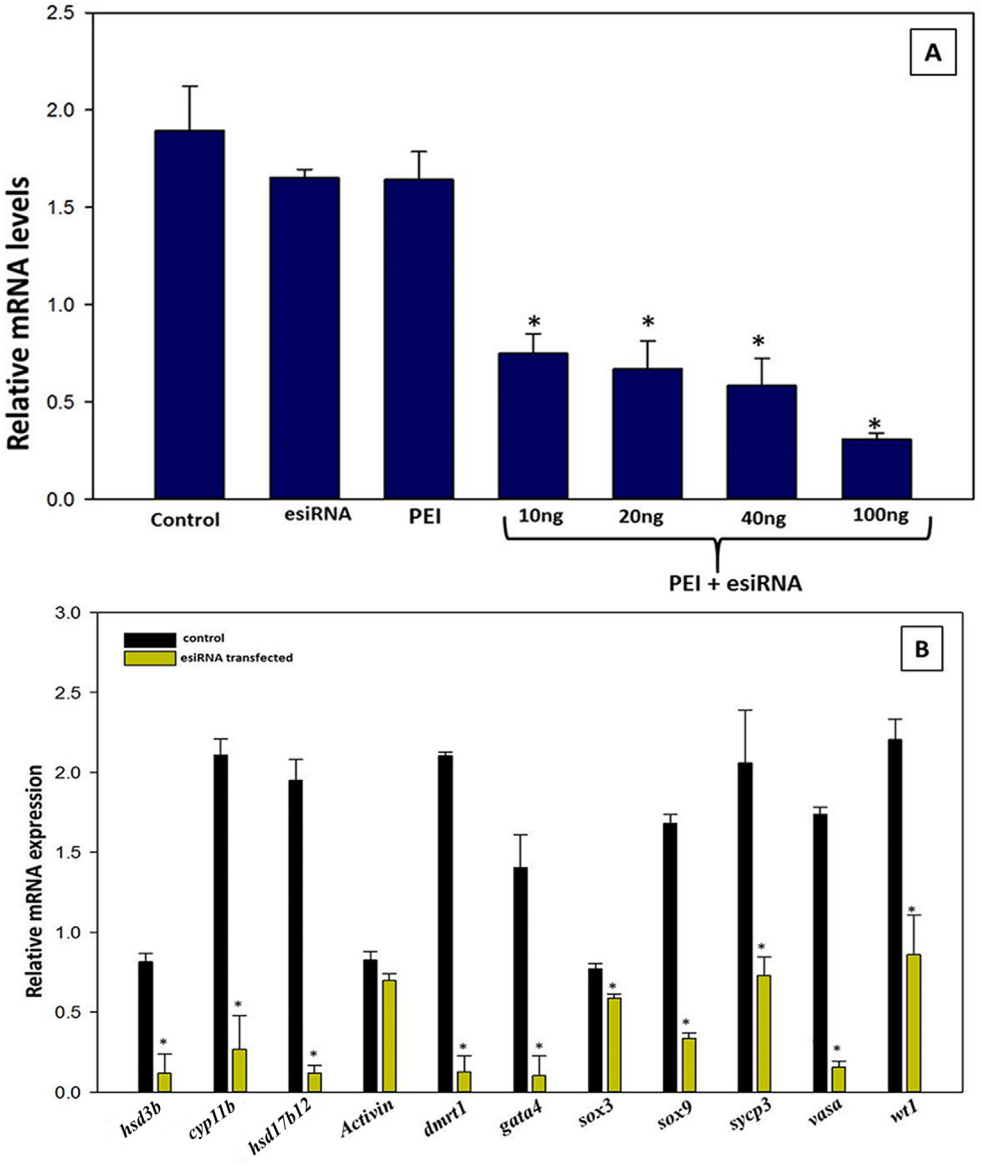


**Fig. 9.** *In vitro* transfection of PEI mediated *c-kit*-esiRNA in catfish testicular culture (mixed; n = 5). Relative expression of *c-kit* was analyzed and was observed to vary with the dose of transfection with *c-kit*-esiRNA (A) and *c-kit* transcripts were quantified and compared between control siRNA, *c-kit*-esiRNA and PEI + *c-kit*-esiRNA groups. Levels of *c-kit* expression were significantly ($P < 0.05$) decreased in the samples transfected by PEI complexed *c-kit*-esiRNA when compared to control groups (A). Expression levels of several testis related factor and steroidogenic enzyme genes (B) were analyzed between control and *c-kit* -esiRNA transfected cultures which showed a significant ($P < 0.05$) decrease in the expression levels when compared to control (*, $P < 0.05$; Mann–Whitney test one way ANOVA on ranks followed by SNK test).

and spermatocytes (Fig. 6A and B) displayed positive signals which revealed a heterogeneous pattern similar to that observed in the IHC analysis (see below) and this localization pattern was constantly seen in most of the testis sections analyzed. Sense probe as a negative control did not show any signal (Fig. 6C), which confirmed the specificity of the antisense probe used.

Localization of c-kit protein using IHC and IF was done in adult catfish testis during late preparatory phase (Figs. 7 and 8, respectively). Pronounced immunoreactivity for c-kit protein (indicated by red arrow-heads) was seen in the developing spermatogonia lining the basement membrane and as well as spermatocytes (Figs. 7A–C and 8A–D). However, the signals were heterogeneous across the lumen, indicating stage specificity. Hence, immunoreactivity was not observed in mature sperm/spermatids counterstained with hematoxylin. Lack of signal in the negative control with c-kit antigen pre-adsorbed antibody of c-kit (Fig. 7D) confirms the specificity. IF detection also displayed a fairly similar result as IHC. Positive signals (FITC stain) were seen in spermatogonia and spermatocytes (indicated by red arrow-heads). Western blot analysis using anti-C-KIT antibody on adult testis sample detected a positive band ( ~ 60 kDa) which corresponds to the cytoplasmic domain of the protein (Fig. 7E).

### 3.5. *In vitro* transfection of c-kit-esiRNA in catfish testicular culture

In the *in vitro* transfection analysis (n = 5), a significant ($P < 0.05$) decrease was observed in the levels of *c-kit* expression in the samples transfected by PEI complexed *c-kit*-esiRNA when compared to control groups (Fig. 9A). The expression levels of *c-kit* after esiRNA transfection also showed varied downregulation with different concentrations. There was a significant ($P < 0.05$) decrease in the expression level of *c-kit* by 78% after transfection with 100 ng/μl of *c-kit*-esiRNA when compared to control, and this dose was used for further experiments. In addition, the transient gene silencing of *c-kit* showed a significant ($P < 0.05$) decrease in the expression levels of other important testis-related factor and steroidogenic enzyme genes when compared to respective controls (Fig. 9B). Transfection of only PEI and *c-kit*-esiRNA separately on catfish testicular cells did not indicate any substantial alteration in *c-kit* expression levels when in comparison with control. The primary testicular culture transfected with control siRNA complexed with PEI were monitored carefully (Fig. 10A and B) and showed fluorescent signals confirming siRNA uptake in the cells (Fig. 10C–F).

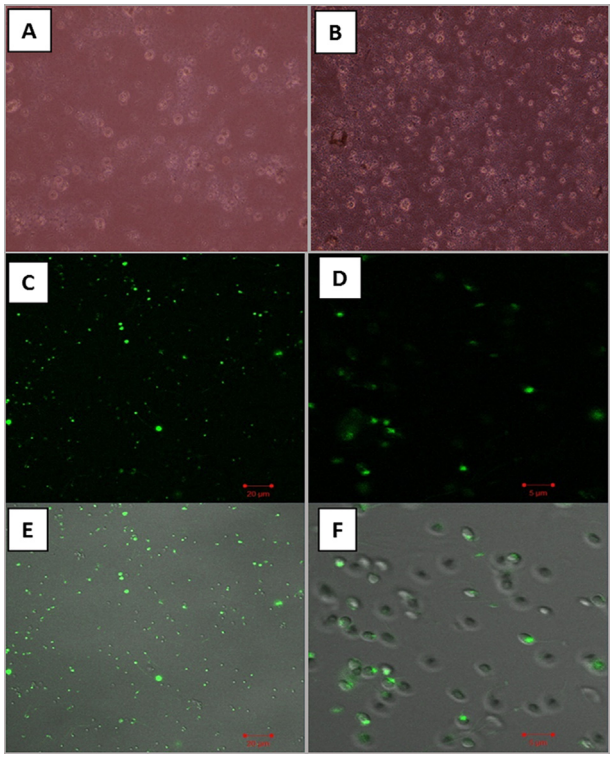


**Fig. 10.** Representative phase-contrast image of catfish primary testicular cells (A and B) and confocal microscope images (C–F) after PEI mediated transfection of control siRNA (MISSION siRNA Fluorescent Universal Negative Control #1, Cat. No. SIC007 conjugated with 6-FAM, Sigma) on catfish testicular culture (mixed) to check the efficiency. Prominent fluorescent signals were observed in the cultured testicular cells, primarily in the spermatozoa.

### 3.6. *In vivo transfection of c-kit-esiRNA in adult catfish*

Initially, PEI alone without any esiRNA was injected directly into the testis of adult catfish and survival of the animal was carefully monitored. Testis samples were then dissected from all transfected groups after 2 and 3 days, individually for quantification of *c-kit* mRNA expression using qPCR. Gene expression levels were measured and compared for PEI, control siRNA (Sigma) as well as *c-kit*-esiRNA-PEI complex injected animals. There were no significant changes observed in the expression levels of *c-kit* in the PEI-only injected catfish (n = 5) when compared to control which infers that neither PEI alone nor control siRNA did negatively alter the gene expression. However, PEI complexed with *c-kit*-esiRNA injected into the testis significantly ($P < 0.05$) decreased the expression of *c-kit* (Fig. 11A). This implied the capacity of PEI complexed *c-kit*-esiRNA in silencing *c-kit* mRNA expression. Quantification of *c-kit* mRNA levels were also done for the tissues collected at two distinct time intervals, 48 and 72 h post transfection and compared to control groups (Fig. 11B). Transfection using *c-kit*-esiRNA combined with PEI caused a significant decrease ($P < 0.05$) in the levels of *c-kit* expression both at 48 and 72 h post transfection when compared to control, with 64 and 57% decrease, respectively. The change in percentage decrease after 72 h indicates that the transcript levels might get restored to normal levels due to transient silencing nature of the experiment as reported earlier by Murugananthkumar and Senthilkumaran (2016). The expression levels of *c-kit* was also measured in kidney to rule out any off-target effects of the transfection, and gene expression levels did not show any significant change (Fig. 11C) indicating the specificity of transient silencing to testis.

In addition, the expression levels of various testis related factors and steroidogenic enzyme genes crucial for germ cell development, were also evaluated with qPCR after 48 and 72 h post esiRNA transfection in the catfish testis as well as in the control groups transfected with PEI conjugated control siRNA (Fig. 12). Interestingly, most of the genes showed a significant decrease ($P < 0.05$) in expression levels at both 48 and 72 h post transfection except for activin β-A where no significant change was evident. The expression levels between 48 and 72 h for most genes remained fairly constant, except for *vasa* whose levels reverted back to normal at 72 h. Western blot was also performed to analyze and confirm the decrease in protein levels of c-kit as well as sycp3 and dmrt1 after transfection (Fig. 13).

### 3.7. *Estimation of serum 11-KT and T levels post c-kit-esiRNA-PEI and control siRNA transfection in catfish*

Levels of serum 11-KT and T measured by EIA were compared between the control and esiRNA-PEI transfected groups (Fig. 14). In the esiRNA-PEI transfected groups, there was a significant but marginal decrease ($P < 0.05$) in the levels of 11-KT (Fig. 14A) when compared to the control, and the same pattern was observed in the levels of T (Fig. 14B) wherein a significant reduction ($P < 0.05$) was evident in comparison to control.

## 4. Discussion

Present study reports the cloning of a cDNA fragment harbouring the signature domain of *c-kit* from catfish, *C. gariepinus* testis using degenerate RT-PCR and RACE strategies. Tissue distribution and reproductive phase expression analysis showed elevated levels of *c-kit* in adult gonads as well as during gonadal recrudescence. The difference in the expression levels between male and females during the pre-spawning phase might be due to the difference in hormonal regulation during this stage. Expression analysis of *c-kit* during embryonic development stages and ontogeny showed elevated expression levels during critical periods of gonadal cycle. IHC and IF revealed the localization of c-kit protein in the spermatogonia and spermatocytes of catfish testis.

Receptor *c-Kit* is a proto-oncogene and its expression has been established in a variety of cells such as germ cells, melanocytes, and hematopoietic cells of several mammalian species (Besmer et al., 1993; Sette et al., 2000; Shin et al., 2014). Extensive studies on its expression and detection have been carried out in mammals such as mouse (Yoshinaga et al. 1991), rat (Wershil et al., 1992), and primates (Galli et al., 1993a,b; Scalercio et al., 2015), but the studies have been extremely limited in lower vertebrates including teleosts as far as current literature is concerned. The present study corroborated with these observations, by initially cloning a partial *c-kit* fragment (1.5 kb) from catfish testis by RT-PCR followed by RACE that includes signature domain regions and a protein band of ~60 kDa molecular mass was also detected with a specific polyclonal antibody against c-kit in the protein lysate isolated from testis, which may correspond to the cytoplasmic terminal part of the receptor or a truncated isoform (Rossi et al., 1992; Albanesi et al., 1996). Tissue distribution analysis demonstrated higher expression of *c-kit* in gonads of both male and female, when compared to moderate expression in kidney and lower in liver and muscle. The role of *c-kit* in gonadal development and gametogenesis has been well documented in humans and mice (Besmer et al., 1993; Lammie et al., 1994; Loveland and Schlatt, 1997; Hutt et al., 2006) as well as in zebrafish (Yao and Ge, 2010, 2013).

The expression of *c-kit* showed a significant increase during preparatory, pre-spawning and spawning phases in males while there is a decrease in the pre-spawning phase in females which might be due to the difference in hormonal pattern and regulation (Senthilkumaran et al., 2004; Martyniuk et al., 2009). The expression was minimal during the resting phase where spermatogenesis is at quiescent stage. In embryonic stages, *c-kit* expression analysis showed a marginal but constant expression up to 20 h and increased significantly at 30 h (the hatched larval stages) which is essentially the period of migration of PGCs to the genital ridge (Nishimura and Tanaka, 2014). Although

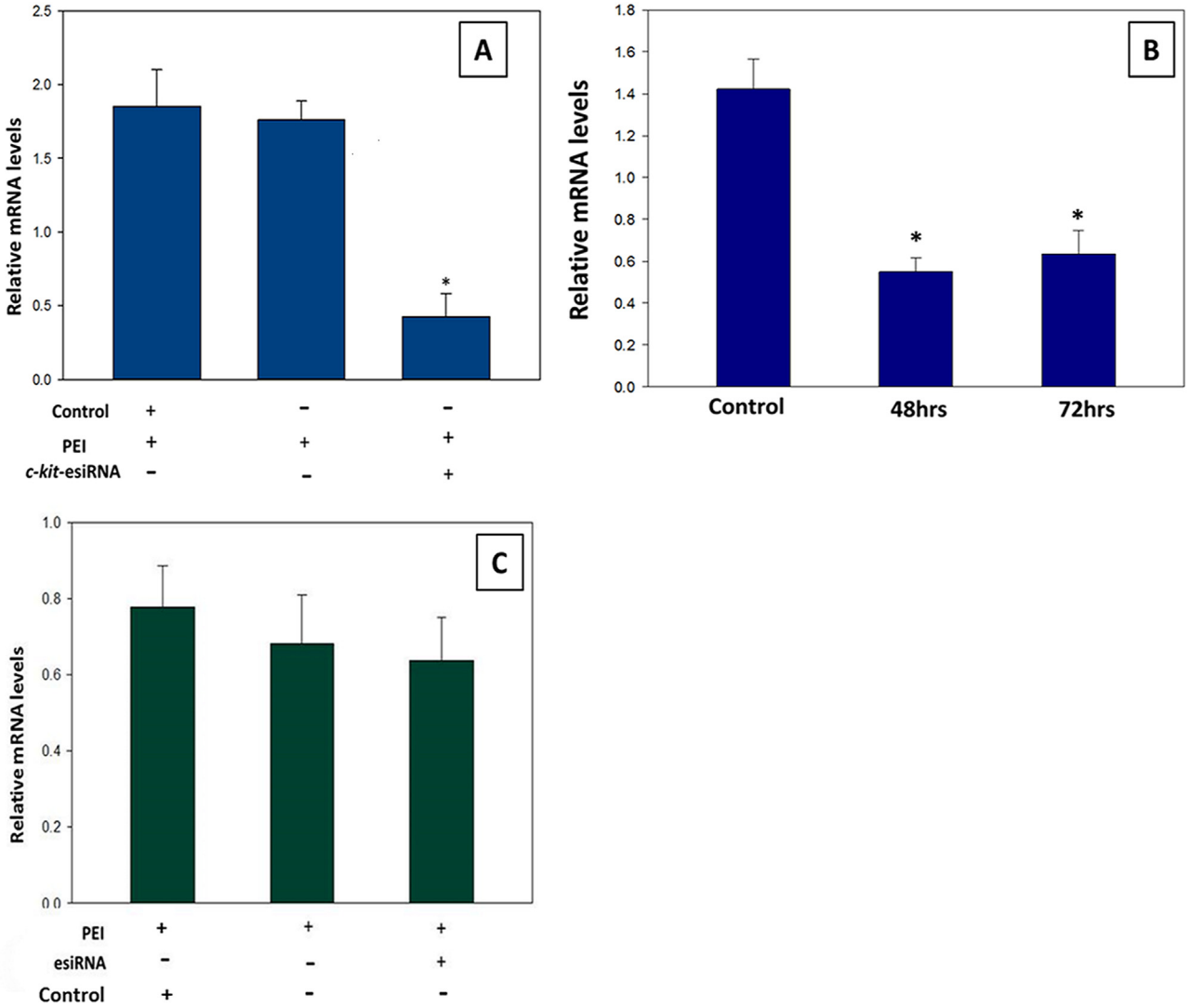


**Fig. 11.** qPCR analysis of the *c-kit* expression levels after esiRNA transfection. (A) *In vivo* PEI mediated *c-kit*-esiRNA transfection in adult catfish (n = 5) which showed that PEI complexed with *c-kit*-esiRNA injected into the testis significantly ($P < 0.05$) decreased the expression of *c-kit*. (B) Analysis of *c-kit* gene expression levels between control and *c-kit* -esiRNA-PEI complex transfected samples at different time points, i.e., 48 and 72 h post transfection where a significant decrease ($P < 0.05$) in the levels of *c-kit* expression was observed post transfection when compared to control, with 64 and 57% decrease, respectively. (C) Expression levels of *c-kit* in kidneys remained constant, ruling out any off-target effects of esi-RNA transfection (*, $P < 0.05$; Mann–Whitney test one way ANOVA on ranks followed by SNK test).

there are limited reports of stage specific *c-kit* expression in teleost species, PGCs have been shown to express *c-kit* in embryonic stages of humans and mice (Manova and Bachvarova, 1991; Pesce et al., 1997; Høyer et al., 2005). In mice embryo, *c-kit* mRNA was detected in PGCs as early as 6.5–7 days post coitus (dpc) which continued during their subsequent proliferation and migration to genital ridge (Manova and Bachvarova, 1991). Various studies have also shown that mutations in the genes encoding either c-Kit or its ligand display abnormal germ cell migration and proliferation during this period (Besmer et al., 1993; Bernex et al., 1996, Chen et al., 2013).

Ontogeny studies at different time points showed marginal levels of *c-kit* expression from 0 to 40 dph, with a dramatic increase from 50 dph, which correspond to the onset and progression of gonadal development and sex differentiation in catfish (Raghuveer et al., 2011). This is also marked by an increase in germ cell population leading to elevated expression of germ cell markers such as *vasa* (Raghuveer and Senthilkumaran, 2010a). This pattern may be partially parallel to the reports on mice where male PGCs, after their arrest at the G0/G1 of the cell cycle at around 13.5 dpc, resume mitotic division again around 3 days after birth which results in a dramatic reduction of *c-kit* expression and can be detected again only in differentiated spermatogonia (Orr-Urtreger et al., 1990; Yoshinaga et al., 1991). In accordance to these findings a similar pattern of expression of *c-kit* was evident in catfish. Further, the dimorphic expression pattern where males display a higher level of expression during 50–75 dph might be due to the difference in the timing of meiosis entry of germ cells in fish (Martínez et al., 2014), where males begin to show a higher proliferation rate of spermatogonia only after ~40 dph as observed in the Japanese medaka (Nishimura and Tanaka, 2014).

The reproductive seasonality and gonadal activity in catfish are under the influence of gonadotropins which also exhibit seasonal pattern (Joy et al., 2000; Kirubagaran et al., 2005). In view of this, the effects of gonadotropins on *c-kit* expression were examined using *in vitro* and *in vivo* induction with hCG. The induction resulted in significant elevation in *c-kit* expression which implied that this gene might also be under the influence of gonadotropins, albeit indirectly through stimulation of Sertoli cells that in turn activate signalling cascades such as BMP4 and retinoic acid to increase *c-kit* levels in the developing germ cells as in mice (Zhang et al., 2011). This contention requires additional studies in teleosts to correlate BMP4 and retinoic acid. Nonetheless,

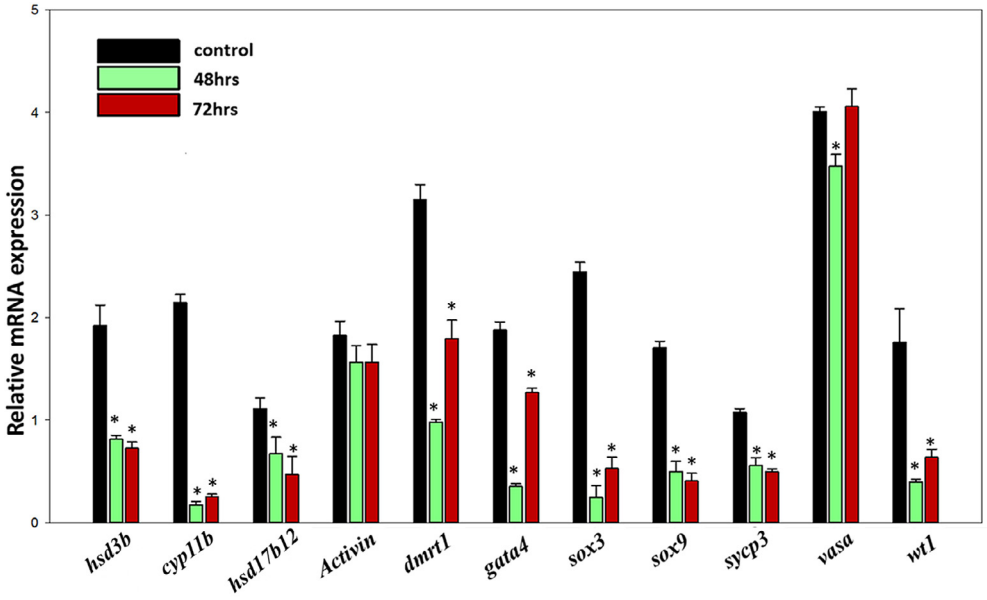


**Fig. 12.** Expression analysis of various testis-related factor and steroidogenic enzyme genes which are crucial for germ cell development and spermatogenesis after *c-kit*-esiRNA transient gene silencing. Most of the genes displayed a significant decrease ($P < 0.05$) in expression levels at both 48 and 72 h post transfection, with the exception of activin β-A which did not show any significant change. Expression levels of each gene are compared to their respective control (*, $P < 0.05$; Mann–Whitney test one way ANOVA on ranks followed by SNK test).

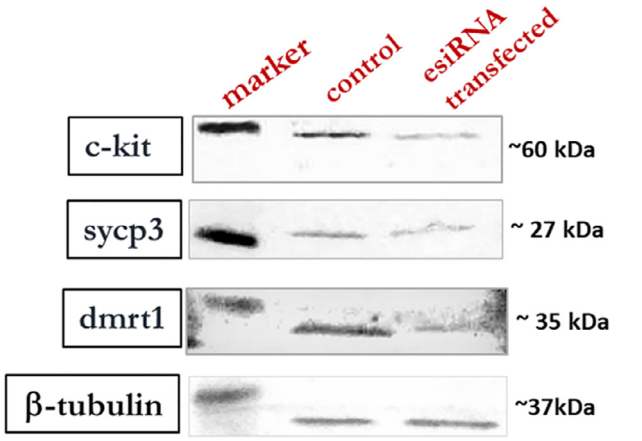


**Fig. 13.** Western blot analysis of protein levels for few genes after esiRNA transfection. The protein levels of c-kit as well as sycp3 and dmrt1 were found to be substantially decreased after esiRNA transfection when compared to control. This reduction in protein levels corroborates the results of the qPCR analysis where most of the genes analyzed showed significant reductions in their expression levels after transfection.

gonadotropin dependency of *c-kit* expression is well validated under *in vitro* and *in vivo* conditions for the first time in any lower vertebrate in this study.

Using an antibody directed against the C terminus, intense immunoreactivity of c-kit was detected in primary and secondary spermatogonia as well as in spermatocytes, but no significant signals were seen in spermatids/sperm in the lumen. A similar pattern of localization was also observed in ISH for *c-kit* transcripts in the mice testis, with the expression mostly seen in developing spermatogonia and spermatocytes (Manova et al., 1990). Additionally, the pattern of signals for the localization experiments showed minor heterogeneity where some areas within the same lumen showed more prominent signals than others. These results were in agreement with localization in mammals where the c-kit receptor expression has been detected in the early spermatogenic cells, but differ in the fact that localization is also seen in the late stages of spermatogenesis particularly in the acrosomal granules in human and rodent testis and sperm (Sandlow et al., 1997; Prabhu et al., 2006; Unni et al., 2009), which is not the case in catfish. To the best of our knowledge based on current available literature, there have been no previous reports of such stage-specific expression of *c-kit* in the spermatogonial cells in teleosts.

The present study also aimed to examine the influence of PEI-

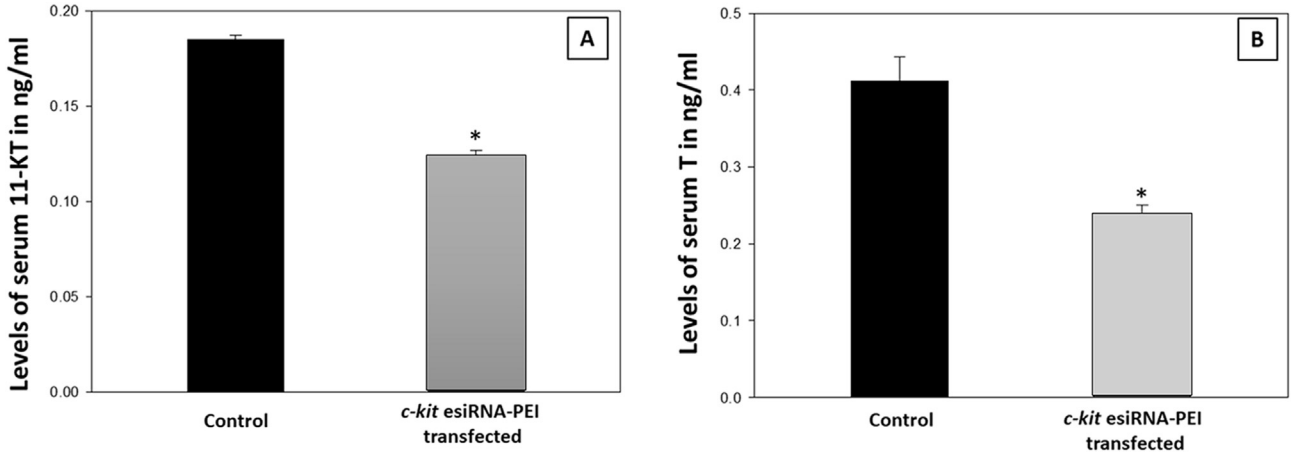


**Fig. 14.** Changes in the levels of serum 11-KT (11-ketotestosterone; inlet histogram A) and T (testosterone; inlet histogram B) post *c-kit*-esiRNA-PEI and control siRNA transfection in catfish. *In vivo* PEI mediated *c-kit*-esiRNA transfection in adult catfish (n = 5) significantly ($P < 0.05$) decreased the levels of serum 11-KT and T. Data (n = 5) were expressed as mean ± SEM. (*, $P < 0.05$; Student's *t* test).

mediated transient silencing of *c-kit* in both *in vitro* and *in vivo* systems. Transient gene silencing effects can be well demonstrated using PEI mediated esiRNA silencing *in vitro* and *in vivo* (Höbel and Aigner, 2010, 2013). The results obtained in the studies using catfish also validate the methodology used earlier with different perspectives (Prathibha and Senthilkumaran, 2016; Murugananthkumar and Senthilkumaran, 2016; Sudhakumari et al., 2017). Transection of *c-kit*-esiRNA via PEI delivery resulted in a significant downregulation of *c-kit* expression levels in both *in vitro* and *in vivo* transient gene silencing. Consequently, the treatment of *c-kit* esiRNA also resulted in the alteration of expression levels of several testis-related factor and steroidogenic enzyme genes critical for germ cell and gonadal development, either directly or indirectly. In mice, siRNA-mediated silencing of *c-kit* in mouse primary spermatogonial cells with anti-*c-kit* siRNA resulted in DNA fragmentation and cell cycle arrest at G(2)/M phase that diminished cell viability and proliferation (Sikarwar and Reddy, 2008). However, knockdown/mutation experiments that explore in detail the impact of the silencing of this gene during gametogenesis is lacking in both mammals as well as teleosts in particular. Down-regulation of germ cell markers crucial for meiosis, *sycp3* (Laldinsangi and Senthilkumaran, 2018) and *vasa* (Raghuveer and Senthilkumaran, 2010a) in catfish, implies the role of *c-kit* in spermatogonial differentiation and meiotic progression in agreement with studies in human and rodents (Rossi et al., 2008; Medrano et al., 2012, Syrjänen et al., 2014). The activation of c-Kit signalling has a decisive role in mice spermatogenesis through meiosis activation at specific time points, which results in the ensuing expression of early meiotic markers such as *Dmc1* and *Sycp3* (Vincent et al., 1998; Di Carlo et al., 2000). The expression levels of *wt1* and *gata4*, genes that are known to play a key role during testicular development of catfish (Murugananthkumar and Senthilkumaran, 2016), were also found to be significantly decreased. Transient gene silencing of *wt1* in catfish have also negatively affected the expression levels of several steroidogenic enzyme genes involved in androgen production (Murugananthkumar and Senthilkumaran, 2016), which may indirectly implicate the involvement of *c-kit* in germ cell development and gametogenesis by affecting important testis-related transcription factors vis-à-vis steroidogenesis either directly or indirectly. This may reflect a similar situation in the down-regulation of other transcription factor genes such as *dmrt1*, *sox3* and *sox9*, whose critical roles have been well documented in gonadal development as well as spermatogenesis in catfish (Raghuveer and Senthilkumaran, 2009, 2010b; Raghuveer et al., 2011; Rajakumar and Senthilkumaran, 2014a). Incidentally, c-kit protein as well as sycp3 and dmrt1 protein levels were also found to be decreased. In addition, *c-kit* transient silencing resulted in decreased expression of several steroidogenic enzyme genes, either directly or indirectly. Although the exact mechanism has not yet been elucidated, aberrations in the expression levels of such key enzyme genes related to testicular function (Raghuveer and Senthilkumaran, 2012; Rajakumar and Senthilkumaran, 2014b, 2015) might indirectly validate the role of *c-kit* in germ cell development and gametogenesis. Downregulation of transcript levels of important steroidogenic enzyme genes such as *hsd3b*, *cyp11b* and *hsd17b12* is further corroborated by the significant reduction in the levels of serum 11-KT and T, the two potent androgens that are principally involved in testicular development and spermatogenesis (Nagahama, 1994). Lack of effect on activin β-A also shows no global effect supporting *c-kit* dependent effect of esiRNA silencing. Activin β-A may have a different mode to enhance spermatogenesis as it is known to induce proliferation of spermatogonia in eel (Miura et al., 1995). Further studies are required to establish the exact cascade of molecular events in relation to *c-kit* interacting genes.

In summary, present report described the cloning, localization and expression analysis of *c-kit* receptor in catfish testis during germ cell development and recrudescence. This may be one of the few detailed reports of this gene in lower vertebrates such as teleosts, specifically in annual breeders. This study implicated the plausible involvement of *c-kit* during the critical stages of gonadal development and gametogenesis via its expression and localization in the germ cells of catfish. The regulatory effects of gonadotropins on *c-kit* were also evident in the *in vitro* and *in vivo* induction studies using hCG. *In vitro* and *in vivo* transient gene silencing of *c-kit* using esiRNA caused a significant downregulation in the expression levels of various testis related genes analyzed, which may infer the role of this gene in testicular germ cell development and gonadal recrudescence, either directly or indirectly.

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## Conflict of Interest

The authors listed in the manuscript have NO affiliations with or involvement in any organization or entity with any financial interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2018.04.004.

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