Involvement of *thoc* gene family and *pentraxin* in the ovarian function of *Cyprinus carpio*

A thesis submitted to University of Hyderabad for the award of the degree

Doctor of Philosophy

In

Animal Sciences

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June, 2019

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CERTIFICATE

This is to certify that the thesis entitled "***Involvement of thoc gene family and pentraxin in the ovarian function of Cyprinus carpio***" submitted by Mr. **Yugantak Raj Gupta** bearing registration number **13LAPH07** in partial fulfillment of the requirements for award of **Doctor of Philosophy** in the School of Life Sciences is a bona fide work carried out by his under my supervision and guidance.

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Parts of this thesis have been:

A. Publications:

1. **Yugantak Raj Gupta,** Durai Sellegounder, Monica Kannan, Seetharam Deepa, Balasubramanian Senthilkumaran, Yaraguntappa Basavaraju. **2016.** Effect of copper nanoparticles exposure in the physiology of the common carp (*Cyprinus carpio*): Biochemical, histological and proteomic approaches. **Aquaculture and fisheries 1:15-23.** (ISSN: 2468-550X, Elsevier); ***(Part of thesis: see Chapter 3)***
2. Arumugam Anitha, **Yugantak Raj Gupta***, Seetharam Deepa*, Manjappa Ningappa, Karani Boraiah Rajanna, Balasubramanian Senthilkumaran. **2019**. Gonadal transcriptome analysis of the common carp, *Cyprinus carpio*: Identification of differentially expressed genes and SSRs. **General and Comparative Endocrinology 279:67-77.** (ISSN: 0016-6480, Elsevier) *(*equally contributed author, partly in chapter 1).*
3. Durai Sellegounder, **Yugantak Raj Gupta**, Raju Murugananthkumar, Balasubramanian Senthilkumaran. **2018**. Enterotoxic effects of *Aeromonas hydrophila* infection in the catfish, *Clarias gariepinus*: Biochemical, histological and proteome analyses. **Veterinary Immunology and Immunopathology 204:1-10.** (ISSN: 0165-2427, Elsevier)
4. Seetharam Deepa, Raju Murugananthkumar, **Yugantak Raj Gupta**, Manjunatha Gowda, Balasubramanian Senthilkumaran. **2019**. Effects of zinc oxide nanoparticles and zinc sulfate on the testis of common carp, *Cyprinus carpio*. **Nanotoxicology 13:240-257.** (ISSN: 17435390, Taylor & Francis Group)

B. <u>Presented in the following conference:</u>

1. ***Humboldt Kolleg on Comparative Endocrinology and Physiology***, *"Expression profiling of thoc during ovarian development in the common carp, Cyprinus carpio"*: **Oral presentation**, 7th-9th January, 2019, Nagpur, Maharashtra, India **(International)**
2. ***6th International Conference on Molecular Signaling (ICMS-2018)***, "*Expression profiling of tho complex (thoc) gene family with reference to ovarian development in the Indian common carp, Cyprinus carpio"*: **Oral presentation**, 8th-10th February, 2018, University of Hyderabad, Hyderabad, India **(International)**
3. ***International Conferences on Reproductive Biology and Comparative Endocrinology (ICRBCE 2017)***, *"Identification of genes/ factors involved in early gonadal differentiation and development using DDRT-PCR in common carp, Cyprinus carpio"*: **Poster presentation**, 9th-11th February, 2017, University of Hyderabad, Hyderabad, India **(International)**
4. ***International Symposium on Comparative Endocrinology and Integrative Physiology (CEIP 2015),*** *"Effects of copper (II) nanoparticles exposure in liver, kidney and gills of common carp, Cyprinus carpio: histological and biochemical analysis"*: **poster presentation**, 4th-7th August, 2015, Thiruvananthapuram, Kerala, India **(International)**

Further, the student has passed the following courses towards the fulfillment of course work required for the award of Ph.D

Course Code	***Name***	***Credits***	***Pass/Fail***
AS 801	Seminar 1	1	Pass
AS 802	Research Ethics & Management	2	Pass
AS 803	Biostatistics	2	Pass
AS 804	Analytical Techniques	3	Pass
AS 805	Lab Work	4	Pass

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DECLARATION

I, **Yugantak Raj Gupta,** hereby declare that this thesis entitled "**Involvement of *thoc* gene family and *pentraxin* in the ovarian function of *Cyprinus carpio***" submitted by me under the guidance and supervision of ***Prof. B. Senthilkumaran*** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this university or any other University or Institute for the award of any degree or diploma.

Date:

Name: Yugantak Raj Gupta

Signature of the Student

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Dedicated to my loving Parents

Acknowledgements

- *At the outset, I express my heartfelt gratitude to my supervisor Prof. B. Senthilkumaran for his outstanding guidance and supervision. His passion for work has been a constant source of inspiration for me, and I will be eternally indebted to him for his undying support and patience.*

- *My sincere gratitude goes to Prof. Aparna Dutta-Gupta for her timely suggestions and invaluable support, as well as her generosity in lending her laboratory facilities whenever required.*

- *I would like to thank my Doctoral Committee members Dr. Sreenivasulu Kurukutti and Dr. Brahmanandam Manavathi for their encouragement, advice and direction throughout the course of my research work.*

- *I express my gratitude to the present Head of Department, Prof. Anita Jagota and former Heads Prof. Jagan Pongubala, and Prof. B. Senthilkumaran, Department of Animal Biology, for providing good equipment facilities in the department.*

- *I thank the present Dean Prof. S. Dayananda and former Deans Prof. KVA Ramaiah, Prof. M.N.V. Prasad, Prof. P. Reddanna, Prof. R.P. Sharma, and Prof. Aparna Dutta-Gupta for access to the school facilities.*

- *I would like to thank all the faculty members of School of Life Sciences for their generosity in providing access to their laboratory facilities.*

- *I thank Dr. C.C. Sudhakumari for her help and support. Her contribution to the laboratory through funds from DST-Women Scientist grant is also greatly acknowledged.*

- *I thank Council of Scientific and Industrial Research, Govt. of India, for the much-appreciated financial support through Junior and Senior Research Fellowships.*

- *I also thank DST, DBT, DBT-TATA, CSIR and UGC for funding our laboratory (Grants provided to Prof. BS).*

- *DST-FIST, DST-PURSE, DBT-CREBB, UGC-SAP/CAS, UPE and University of Hyderabad funding to the Department and the School are also acknowledged.*

❖ *I also express my sincere gratitude to my present and former lab-mates, Dr. A. Rajakumar, Dr. Y. Prathibha, Dr. Murugananthkumar, Dr. S.K. Mamta, Dr. S. Durai, Dr. J. Varshini, Dr. Anshu Sharma, Ms. S. Deepa, Ms. A. Anitha, Ms. Sonika Kar, Ms. Akanksha Pranoty, Ms. T. Swathi, Ms. N. Anusha, Mr. S. Prabhakar, for all their all invaluable support, both on the academic and personal fronts. All the experiences I encountered and life lessons that I have learned from them will always be cherished.*

❖ *I thank all my friends especially, Ms. Kalyani and research scholars especially, Ms. Doris, Ms. Sheetal, Mr. Kowshik, Mr. Naren, Dr. Vinod of SLS for their timely help.*

❖ *I thank Mrs. Leena Bashyam, Mrs. Monica Kannan and Mrs. Nalini for their help in Genomics, Proteomics and Confocal microscope facilities, respectively.*

❖ *I thank all the non-teaching staff in the School, Department and Animal house for their help and all our lab attendants for the maintenance of the laboratory and aquaculture facilities.*

❖ *I thank the University of Hyderabad for providing access to extensive online resources in the form of journal subscriptions as well as internet facility provided via the CNF that has aided my research work every step of the way.*

❖ *My sincere gratitude to my best friends, who have lent their ears, shoulders and hearts without hesitation through all these years, and I am a better person because of them.*

❖ *Words are inadequate to describe the love and gratitude I have for my family, whose boundless support and encouragements have made everything possible for me to get to where I am now.*

.

~Yugantak Raj Gupta~

Abbreviations used in thesis

ad4bp/sf-1	:	Adrenal 4 binding protein/steroidogenic factor-1
amh	:	Anti-Müllerian hormone
BCIP	:	5-bromo-4-chloro-3-indolyl phosphate
BSA	:	Bovine serum albumin
CA	:	Cortical alveoli
CREB	:	Camp responsive element binding protein
CRP	:	C reacting protein
CV	:	Cortical vesicles
cyp19a1a	:	Cytochrome P450, family 19, subfamily A, polypeptide 1a
DAB	:	3′, 3′-diaminobenzidine
DDRT-PCR	:	Differential display reverse transcription- PCR
DIG	:	Digoxigenin
dph	:	Days post hatch
dpt	:	days post treatment
EDs	:	Endocrine disruptor
EIA	:	Enzyme immunoassay
esr1	:	Estrogen receptor 1
esr2	:	Estrogen receptor 2
FBS	:	Fetal bovine serum
FCM	:	Flow Cytometry
fdzR	:	Frizzled receptor
fgf9	:	Fibroblast Growth Factor 9
foxl2	:	Forkhead box L2
FS	:	Forward light scatters

FSH	:	Follicle stimulating hormone
GCs	:	Granulosa cells
gdf9	:	Growth differentiation factor 9
GTH	:	Gonadotropin
hsd11b1	:	Hydroxysteroid (11-β) dehydrogenase 1
hsd17b1	:	Hydroxysteroid (17-β) dehydrogenase 1
hsd20b	:	Hydroxysteroid (20-β) dehydrogenase
hsd3b	:	Hydroxy-Δ-5-steroid dehydrogenase, 3β
igf1	:	Insulin-like growth factor-1
IHC	:	Immunohistochemistry
*IS*H	:	*In situ* hybridization
kDa	:	Kilodalton
LH	:	Luteinizing hormone
M199	:	Medium199
MS 222	:	Ethyl 3-aminobenzoate methane sulfonate
NP	:	Nanoparticle
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction
PEI	:	Branched Polyethylenimine
PFA	:	Paraformaldehyde
PGC	:	Primordial germ cells
pou5f3/oct4	:	POU domain, class 5, transcription factor 3/octamer-binding transcription factor 4
qPCR	:	Quantitative real-time PCR

RACE	:	Rapid amplification of cDNA ends
rspo1	:	R-spondin 1
RT	:	Room temperature
SAP	:	Serum amyloid P component
sox9b	:	SRY-related high mobility group (HMG) box 9b
SS	:	Side light scatters
star	:	Steroidogenic acute regulatory protein
TBS	:	Tris-buffered saline
TCs	:	Theca cells
thoc	:	THO complex
vtg	:	Vitellogenin
17α,20β-DP	:	17α,20β- dihydroxy pregnen-3-one
17β-E_2	:	17β-estradiol

All the gene name for the teleosts are written in *lowercase italics* while for mammals First letter of gene is in *uppercase* and others are in *lower case italics* in thesis.

Contents

General Introduction

Oogenesis is a dynamic and ordered process, in which oocytes undergo a series of developmental or growth processes till meiotic maturation to ensure sufficient quantity of mature eggs for fertilization. During this process, several gene/factors which have a crucial role, are differentially expressed and coordinated to give rise to fully–developed haploid oocytes. In general, oogenesis process is a series of events regulated by several genes/ transcription factors starting from the formation of PGCs, otherwise known as germline segregation. PGCs further undergoes ovarian determination/differentiation by transforming into oogonial cells to initiate meiosis after rapid mitotic division. The oocytes then undergo growth phase to enter meiotic arrest, which eventually resumes during final oocyte maturation or puberty. Lastly, it culminates in ovulation by expulsion of an ovum from follicles (Patiño and Sullivan 2002). In addition to PGCs, several other factors also complex the oogenesis process such as synthesis and processing of yolk protein in developing oocytes, subsequently, vitamin deposition and egg envelop formation during oocytes maturation. (Wallace and Selman 1990; Patiño and Takashima, 1995). Several pioneering works have been done on teleost reproductive physiology, especially in female reproductive system (Nagahama, 1983; Bhattacharya et al., 1990; Haider and Balamurugan, 1995; Nakamura et al., 1998; Senthilkumaran and Joy, 2001; Nakamura et al., 2011; Maitra et al., 2014), yet, there is scarce of information to understand all dynamic processes associated with the oogenesis.

Similar to higher vertebrates, ovarian follicle is a functional unit in teleost as well and it consists of an outer TCs and an inner GCs layer. During the process of oocyte development, also known as folliculogenesis, these cells undergo proliferation process to give rise a GCs monolayer (Kagawa 2013). Ovarian differentiation of germ cell stage to oocyte development

in teleosts differs from the mammals at structural and functional levels in oogenesis and reproductive behavior. Further, seasonal breeding involving a specific phenomenon of gonadal recrudescence is essentially important in various teleost species inhabiting subtropical climate regions. A better understanding of regulation of these molecular and functional events during ovarian development will provide better insights.

Ovarian Development

As mentioned, primary follicles consist of pre-follicular cells (presumptive GCs) which undergo extensive proliferation during the process of folliculogenesis at the onset of meiosis. In ovary, GCs surrounds the oocytes and TCs present over the basement membrane (Fig. 1). This structure remains essentially unchanged throughout follicular growth (Tokarz 1978; Wallace and Selman 1990; Patiño and Takashima, 1995).

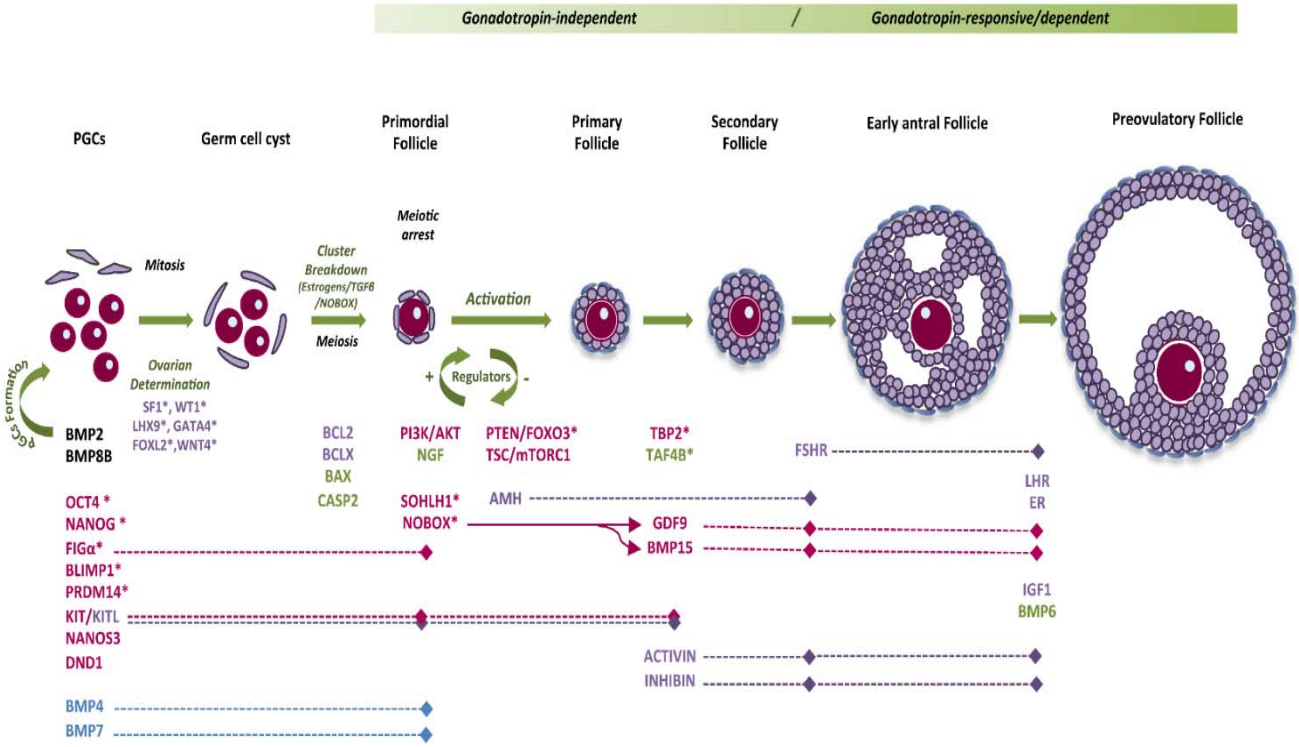

Fig. 1: A schematic description of oocyte developmental stages about meiosis in teleost fish. Adapted from Sánchez and Smitz (2012).

Further, follicular growth can be generally classified into previtellogenic and vitellogenic stages. During vitellogenic growth, exogenously synthesized (predominantly from liver) yolk protein starts to accumulate within oocytes resulting in the expansion of ovarian follicle. In this process, large amounts of ribosomal and heterogeneous RNA are produced by nucleoli located at the periphery of germinal vesicle (nucleus) of the oocyte. Indeed, much of the mRNA present in mature oocytes are produced during previtellogenic stage (Wallace and Selman, 1990). The oocyte content of specific mRNAs, such as *vtg* processing enzyme mRNAs, and Vtg receptor, appears to peak during previtellogenic growth and decline subsequently. Vtg is a large (300–600 kDa) glycophospholipoprotein synthesized by the liver and taken up by growing oocytes (Wallace, 1985; Mommsen and Walsh, 1988).

Along with *vtg* mRNA, there are also the synthesis of glycoproteins (polysialoglycoproteins) during mid- to late-previtellogenic growth (Wallace and Selman, 1990) and incorporated into alveoli newly formed at the oocyte's periphery. These are known as cortical alveoli because of their initial location, and they grow in size and number and then decreases during late vitellogenic growth as yolk accumulates centripetally in the oocyte (Blazer, 2002). At the time of fertilization, CA discharged its content into space between oocyte and the or vitelline envelope (zona radiata or chorion). In teleost, several isoforms of *vtg* genes or proteins were identified several fish species such as zebrafish and rainbow trout (Matsubara et al., 1999; Trichet et al., 2000; Wang et al. 2000; Reith et al., 2001). Vitellogenesis is the process by which Vtg protein was synthesized in liver and transported to developing ovarian follicle of fish. During this several hormones regulate the process of vitellogenesis such as E_2 (van–Bohemen et al., 1982). Environmental contaminants that mimic estrogen can induce vitellogenesis,

even in immature or male fish, and thus vitellogenesis is used in fishes as a biomarker of exposure to 'environmental estrogens' (Tyler et al., 1999). Other natural hormones and growth factors may also modulate hepatic vitellogenesis.

Molecular basis of ovarian development and function

Sex determination is the initial process to determine the fate of bipotential gonad. Unlike mammals, in teleosts, various factors (both genetic or environmental) involve together at pre–gonadal stage to regulate the process of sex determination or gonad determination (Kobayashi and Nagahama, 2009; Siegfried, 2010; Uller and Helanterä, 2011). Subsequently, the presence of hierarchical nature, a single gene or environmental cue drives gonad developmental pathway towards one direction or another. In successive events, germ cell proliferation rate can also be an important factor. During this period, the germ–somatic cell crosstalk is critical, yet largely uncharacterized. (Fig. 2)

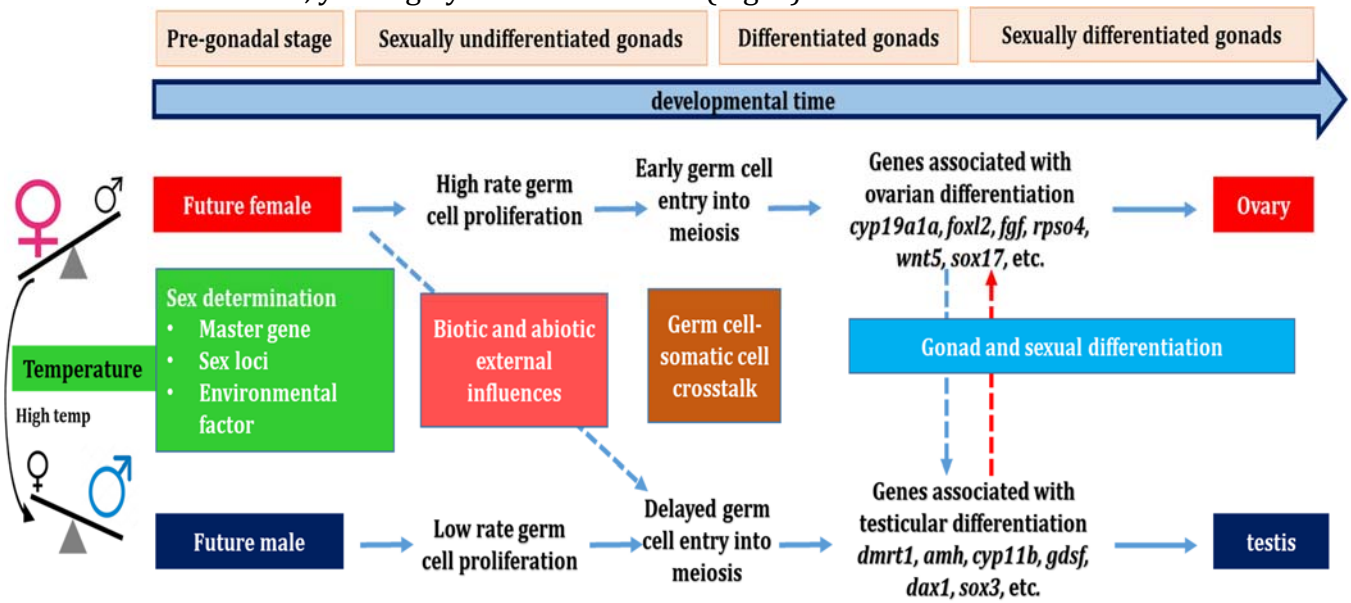

Fig. 2: Major events leading to ovarian versus testicular differentiation in fish. Adapted (modified) from Martínez et al. (2014).

After gonad determination, a network of transcription factors interacting together at different ovarian developmental stages, which are influenced by hormones and ligands (Huang et al., 2017). These putative interactions were observed in mammals from the early gonadal differentiation to the oocyte development by using several knockout models as represented in Fig. 3 (Garcia–Ortiz et al., 2009).

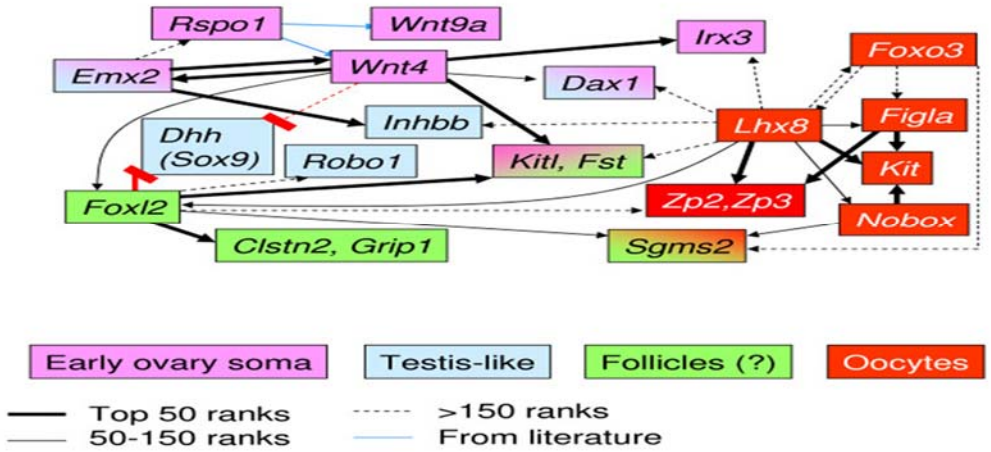

Fig. 3: Schematic representation for the putative interactions inferred from the assessment of several knockout models.
Adapted from Garcia–Ortiz et al., 2009.

Gonad differentiation is well studied in mammals where its initiation and maintenance by distinct pathways involving multiple genes, such as *Foxl2*, *Rspo1*, *Ctnnb1*, and *Wnt4* that work synergistically to ensure ovarian development (Eggers et al., 2014). In the case of teleosts, studies on ovarian development at molecular level is limited to very few transcription factors and their interactions (Sudhakumari and Senthilkumaran, 2013). Some of the reports showed that at gonad development early stages, many genes interacting predominantly each other such as *sox9* and *fgf9* expression regulating *wnt4/β–catenin* expression (Nef and Vassalli, 2009). At the same time, high expression of ovarian aromatase (*cyp19a1a*) plays a vital role in ovarian growth. *cyp19a1a* is predominantly responsible for the balance between

androgens and estrogens, by the action of other genes or environmental factors like temperature (Navarro–Martín et al., 2011) or the interaction between the *amh* and its receptor, which triggers an essential signaling pathway for testis development (Kamiya et al., 2012). Ovarian cyp19a1a belongs to the cytochrome P450 superfamily (CYPs), a terminal enzyme in the steroidogenic pathway responsible for the conversion of androgens to estrogens (Simpson et al., 1994). It plays an essential role for the regulation of ovarian differentiation, vitellogenesis and sex change in most teleosts as it is ovary specific (Chourasia and Joy, 2008; Guiguen et al., 2010; Rasheeda et al., 2010). It is known to be up–regulated by *ad4bp/sf–1* along with co–activator *foxl2,* in the Nile tilapia (Yoshiura et al., 2003; Wang et al., 2007). Various transcription factors are known to play an essential role in ovarian development of teleosts other than *cyp19a1a* as represented in figure 4.

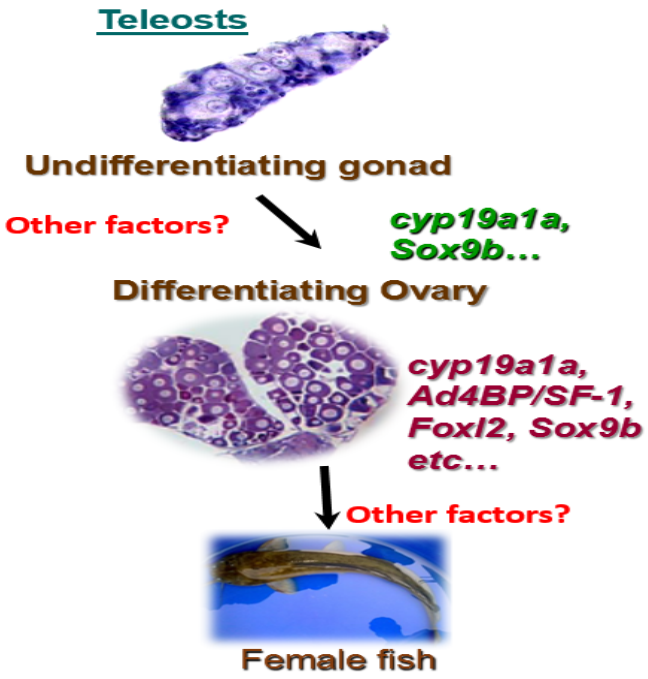

Fig. 4: Schematic representation of roles of genes evaluated in ovarian development of teleosts.
Adapted as reviewed in Sudhakumari and Senthilkumaran, 2013.

Subsequent section will highlight critical genes related to ovarian growth and development.

ad4BP/sf–1

A member of orphan nuclear receptor protein NR5A1 superfamily can also act as a transcription factor. It is crucial for endocrine hormone production in gonads and adrenal glands as well as in the regulation of steroid hormone biosynthesis (Morohashi et al., 1992; Parker et al., 2002). It plays an important role in the transcriptional regulation of CYP genes in mammals (Morohashi and Omura, 1996). The significance of *ad4bp/sf–1* in teleostean CYP genes has been dealt earlier.

foxl2

A forkhead transcription factor, is one of the earliest ovarian markers and preferentially expressed from an early stage of ovary to regulate gonadal differentiation, steroid metabolism, and various other developmental processes (Sridevi and Senthilkumaran, 2011; Georges et al., 2013). It interacts with *ad4bp/sf–1* to up–regulate *cyp19a1a* gene transcription in tilapia (Wang et al., 2007) and is also known to repress the gene that codes for a *star* at transcriptional level.

sox9b

SOX9, transcription factor, a member of the SOX superfamily of genes. *sox9b*, *sox9* ortholog, has specific significance to gonadal differentiation in females. Interestingly, *sox9* or its isoform also plays a crucial role in testicular differentiation (Kent et al., 1996; Raghuveer and Senthilkumaran, 2010).

Wnt signaling molecules

Wnts comprise a family of signaling molecules and play a critical role during ovarian follicle development in mammals (Boyer et al., 2010). They are one of the essential signaling pathways required for various developmental processes during embryogenesis. Wnt signaling molecules impart dynamic changes in mammalian ovary during the reproductive cycle (Hernandez–Gifford, 2015). Wnt4, the member of Wnt family, having a role in granulosa cell expansion throughout folliculogenesis process in rodents (Hsieh et al. 2002: Hernandez–Gonzalez et al. 2006). Recent study from our laboratory demonstrated a critical role for *wnt4/5* in teleostean ovarian development (Prathibha and Senthilkumaran, 2017). Few other studies in teleosts also endorsed the regulatory role of Wnt family (Nicol and Guiguen, 2011; Wu et al., 2016)

Though ovarian development is a complex process, involvement and function of various other factors are largely unknown except for the genes mentioned above and their interactions. Genes which were essential for ovarian development in mammals were neither identified, nor expression pattern was explored exhaustively in teleosts. It is necessary to investigate other essential genes/molecules for high throughput breeding practices as well as to understand the process of gonadal development till maturation. In this context, few studies in the Japanese eel used various strategies to improve seed production (Kagawa et al. 2013). Similar efforts are not done in variety of Indian habitat fish models.

Endocrine axis and steroidal regulation of ovarian development

In addition to regulation by transcription factors/genes, endocrine axis and steroids do have a critical role during ovarian development. Two types of pituitary gonadotropin (GTH–I and GTH–II) homologous to FSH and LH in mammals have been identified in teleosts (Swanson

et al., 1991). These mediate the events oocyte growth and maturation through sex steroid synthesis which is under the regulation of gonadotropin–releasing hormone. In teleosts, genes encoding GTHs, FSH, and LH, are differentially expressed during secondary ovarian follicle development (Swanson et al., 2003; Yaron et al., 2003; Levavi–Sivan et al., 2010). There are several studies on GTH's significant role during vitellogenesis and ovarian maturation. During secondary ovarian development process, elevated FSH levels trigger follicular steroidogenesis, resulting in increased E_2 production. This elevated level of E_2 further stimulates the synthesis of hepatic Vtg and newly synthesized Vtg then incorporated by a receptor-mediated process into the follicles (Lubzens et al., 2010). On the other hand, LH levels increase just before final oocyte maturation and ovulation which perhaps facilitated by low levels of E_2 with concomitant rise in 17α, 20β–DP (Breton et al., 1998; Joy et al., 1998; Lubzens et al., 2010; Kagawa et al., 2013). In–depth analysis of GTH receptors has been done using a variety of fish models explicitly (Kazeto et al., 2012; Nyuji et al., 2015). GTHs (FSH and LH) acts on theca and granulosa cell layers for regulating ovarian vitellogenesis and maturation is termed as "two-cell and two GTH" hypothesis (Nagahama, 1983). GTHs exert action on TCs and GCs layers for production of steroid hormones, E_2 to maturation–inducing hormone, 17α,20β-DP as well as the steroidogenic enzyme genes from *cyp19a1a* to *hsd20β* that mediate vitellogenesis and final oocyte maturation (Senthilkumaran et al., 2004). During vitellogenesis, Foxl2 and Ad4BP/SF-1 together transcriptionally regulate the expression of *cyp19a1a* and converts testosterone to E_2 by GCs. On the other hand, during oocyte maturation process, LH induces shift in steroidogenesis from E_2 to 17α,20β-DP as well as the steroidogenic enzyme genes from *cyp19a1a* to *hsd20β* along with transcriptions factors shift

of AD4BP/SF–1 to CREB (Fig. 5). Both the processes, oocyte growth (vitellogenesis) and ovarian maturation is governed through CREB in the GCs layers of ovarian follicles, however, ovarian growth is predominantly regulated by AD4BP/SF-1 and Foxl2 (Senthilkumaran et al., 2002, 2004; Sreenivasulu and Senthilkumaran, 2009; Murugananthkumar and Senthilkumaran, 2013, Senthilkumaran et al., 2015).

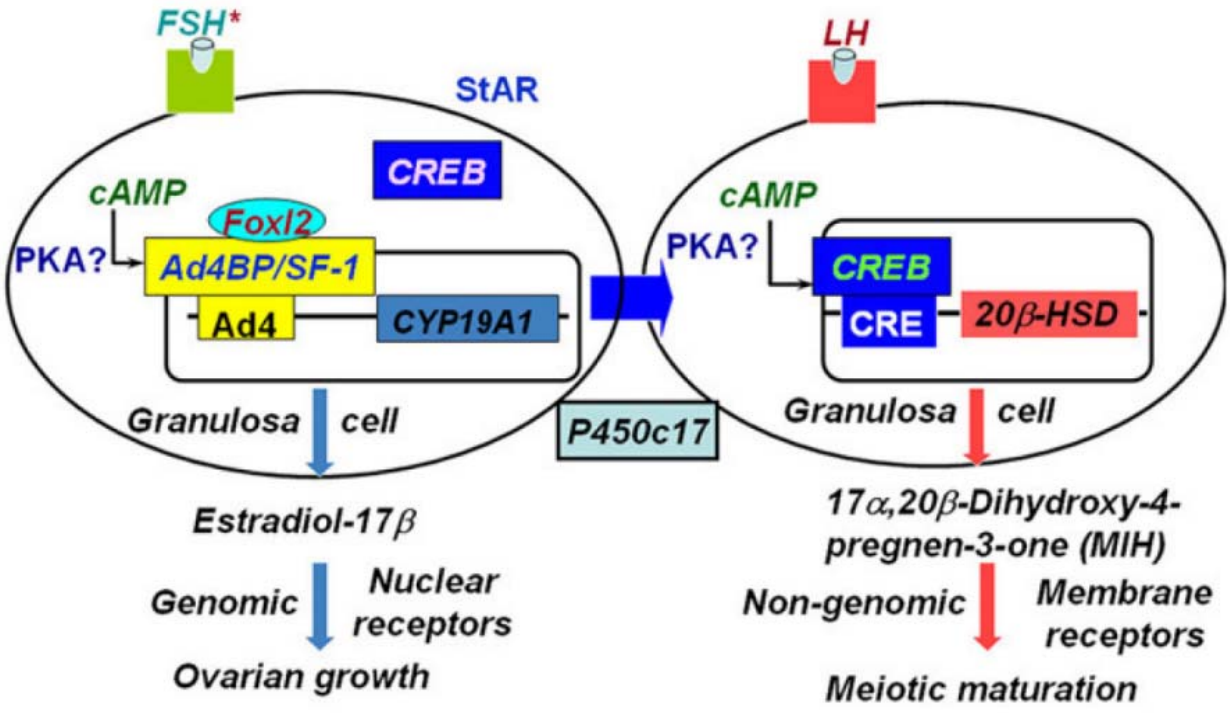

Fig. 5: Transcriptional regulation and shift in steroidogenesis in granulosa cells of ovarian follicles undergoing oocyte growth and maturation.
Adapted from Senthilkumaran et al., 2004

In contrast to extensive work on ovarian growth, vitellogenesis, and maturation (Nagahama, 1997; Senthilkumaran et al., 2002, 2004; Clelland and Peng, 2009; Sreenivasulu and Senthilkumaran, 2009; Senthilkumaran et al., 2011; Murugananthkumar and Senthilkumaran, 2013; Maitra et al., 2014; Senthilkumaran et al., 2015), endocrine regulation during primary ovarian growth and previtellogenic secondary follicle entry is relatively less

understood. Irrespective of the precise roles of GTHs, steroid production and its subsequent signaling are critical for promoting primary and early secondary ovarian follicular growth, oocyte maturation, and ovulation (Jamnongjit and Hammes, 2006). In teleosts, during sexual developmental process, steroidogenic enzymes and related transcription factors expression is a stage– and tissue–specific (Raghuveer et al., 2011; Rajakumar and Senthilkumaran 2014). Drummond (2006) showed that steroid action has a crucial role at the various developmental stages of the ovarian follicle and ovarian function, and its absence can cause infertility. In mammals, few components of female pathways like Wnt4 and Rspo1 are known to involve in steroidogenesis by suppressing Leydig cell development (Kocer et al., 2009) as shown in figure 6 whereas, in the case of teleosts, these aspects are not yet explored.

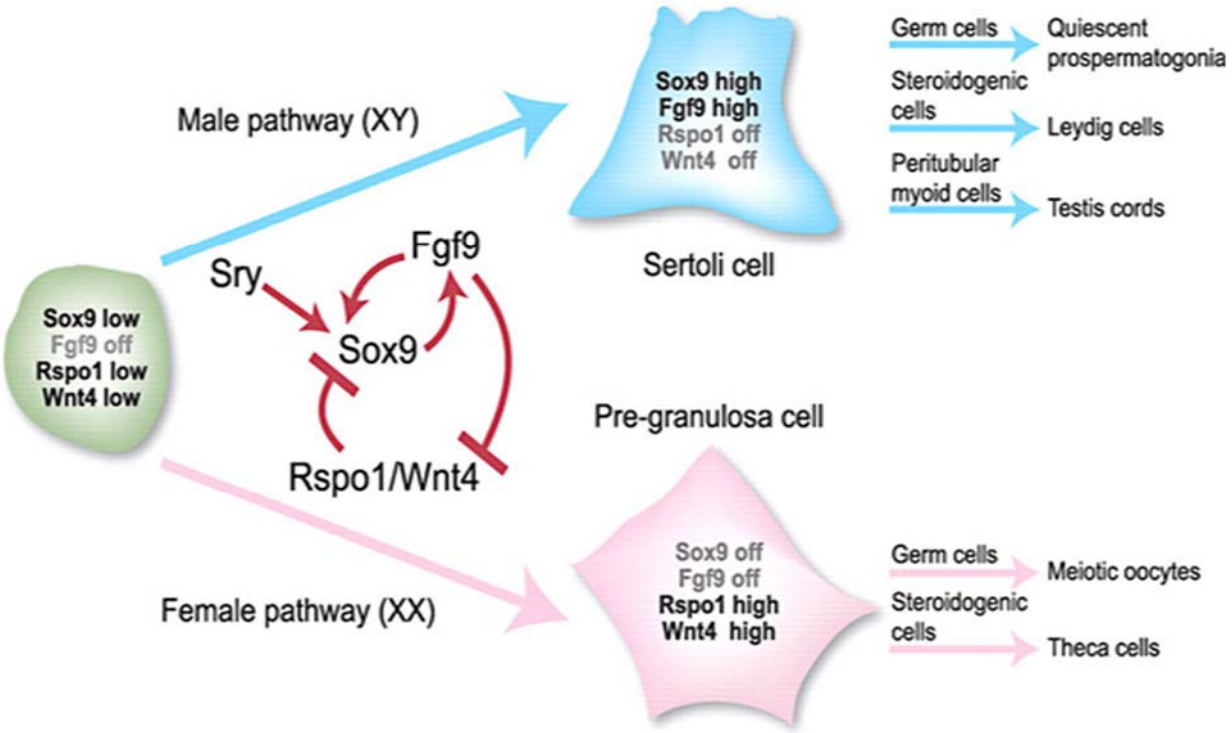

Fig. 6. Mouse-gonadal supporting cell development
Adapted from Kocer et al., 2009.

Taken together, it is obvious that various genes/factors play a crucial role to regulate critical events of oogenesis. Incidentally, gonadal development is altered by EDs of different kinds and hence it is imperative to understand the molecular mechanism of endocrine disruption.

Effects of ED on ovarian development

Unlike mammals, fishes display a diverse classification of gonadal development through physiological, behavioral, and ecological adaptations having sexual plasticity (Shapiro, 1992; Baroiller and D'Cotta, 2016). Sex determination and gonadal differentiation are influenced by genetic, physiological, and environmental factors (Devlin and Nagahama, 2002; Kobayashi and Nagahama, 2009; Siegfried, 2010; Uller and Helanterä, 2011; Huang et al., 2017). Environmental factors, such as temperature, photoperiod, or salinity, have a significant influence on this process (Strüssmann et al., 2002). However, in teleosts, gender of the fish is not morphologically defined during pre-gonadal stage, as it does not have gonads differentiated into testis or ovary (Devlin and Nagahama, 2002). At this stage, teleost gonads can be manipulated, and one of the ways to manipulate the sex of fish is the use of ED. It is well established that EDs mimic the natural action of hormones at several stages leading to the obstruction of homeostasis and developmental regulation of organism. Most commonly used EDs are exogenous steroid hormones, and another mode is to impart endocrine disruptor using pesticide, hormone mimic, or NPs of trace elements. Unfortunately, use of NPs may not be a consistent method to culture mono–sex population. In our approach using NPs, for example, Cu-NPs cause oxidative stress (*see chapter 3) and disrupted testis development (Murugananthkumar et al., 2016). Incidentally, steroid, hormonal, or steroid

hormone mimic are highly preferred to produce physiologically functional mono–sex population stress (*see chapter 3). There are several reports where androgen or E_2 administered in immersion baths or by feed during pre–gonadal stage resulted in skewing into the mono–sex population as per sex steroidal class and dose (Rougeot et al., 2002; Flynn and Benfey, 2007; Arslan et al., 2009; Raghuveer and Senthilkumaran, 2009). This kind of approach will be good to understand the function of gonad–specific genes while discovering essential role in gonadal development like the aim of the present work.

Common carp as an animal model

In the present study, *Cyprinus carpio*, commonly known as common carp, was chosen as an animal model, and they are an excellent model for genomics studies. It follows a biannual seasonal reproductive cycle or recrudescence which has four stages as represented in Preparatory, Pre–Spawning, Spawning, and Post–Spawning as described below.

C. carpio is a widespread, highly desirable freshwater food fish in India. International Union for Conservation of Nature declared *C. carpio* as a vulnerable species poised for extinction. Common carp is a biannual breeder yet occasionally have even three spawning periods annually, which are linked to the start of rainy season and floods. It belongs to the most evolved infraclass Teleostei of order *cypriniformes*, under the bony fishes. Teleost group ranging from hermaphroditism to gonochorism exhibit a broad range of sexual plasticity and also, they have various level of regulation during the sex determination i.e. from genetic factors to environmental cues. Further, there is studies showed the sex of fish can be manipulated or completely reversed by exogenous steroid treatments exposing during critical windows of sex determination/differentiation, their sex can be manipulated or

completely reversed. Lack of blood barrier in organs, for example, at the level of brain, pituitary, testis, and ovary makes it simplified system for several experimental manipulations. Common carps have most of the characteristics mentioned above and it also undergo seasonal pattern of gonadal maturation and recrudescence, which provide a chance to analyze sexual maturation events more than once in its reproductive cycle. In turn, they also serve as useful models to provide an insight into understanding the expression pattern of sex-specific genes during gonadal development and recrudescence (Fig. 7).

Kingdom	:	Animalia
Phylum	:	Chordata
Class	:	Actinopterygii
Order	:	Cypriniformes
Family	:	Cyprinidae
Subfamily	:	Cypininae
Genus	:	*Cyprinus*
Species	:	*carpio*

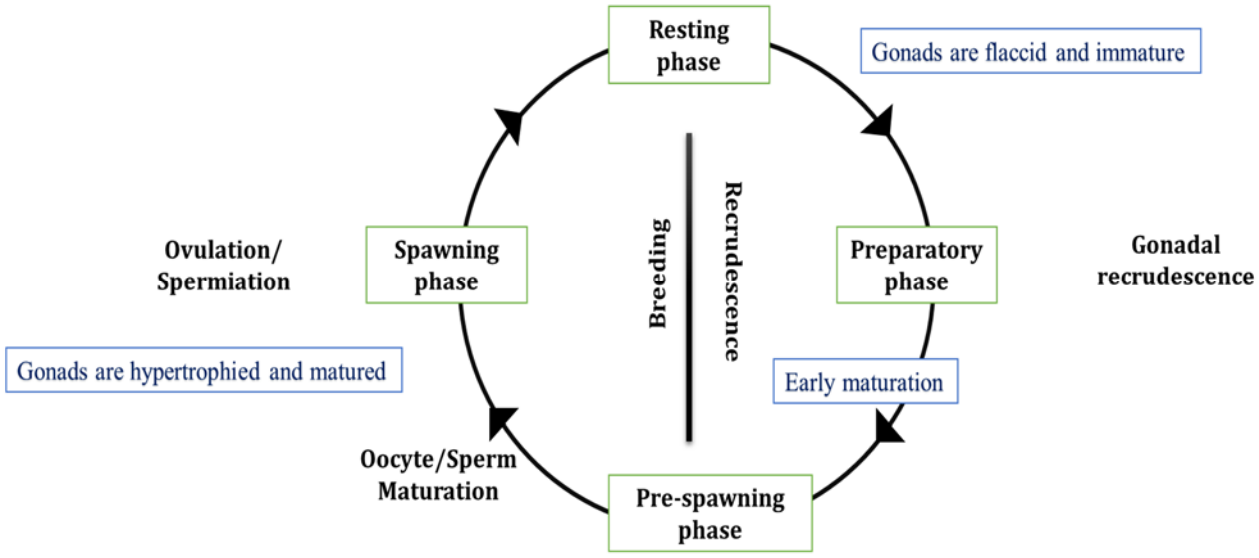

Figure 7 depicting common carp, classification and reproductive cycle.

DDRT

In order to identify novel genes towards the understanding of teleostean oogenesis, which is the major aim of this study, it is essential to isolate and characterize differentially expressed genes as a first step. Differential display (also referred to as DDRT-PCR or DD-PCR) is a gel-based powerful technique to compare and identify differentially expressed genes (Peale and Gerritsen, 2001, Moustafa et al., 2016). In DDRT-PCR, short arbitrary primers were used in combination with anchored oligo-dT primers to amplify 3' termini of mRNA. (Liang and Pardee, 1992). In DDRT, prior information of genes does not require; the method is an “open” system with a potential to detect most of the genes expressed in a cell even low abundance transcripts (Venkatesh et al., 2005; Moustafa et al., 2016; Yu et al., 2018). DDRT–PCR strategy is the combination of three main techniques performed sequentially: starting with reverse transcription of mRNA of each sample using a set of 3′ "anchored primers" (short sequence ending with oligo-dT) for cDNA synthesis for each sample. Followed by PCR amplification of each sample using another set of primer i.e. 3′ arbitrary primer which identical to anchored primes those used during cDNA synthesis. After PCR, amplicons were electrophoresed and visualized in 6% Urea gel for the further analysis. In DDRT, there are two sets of primers, i.e., Anchor primer (Four in number HT11-A/C/G/T) and arbitrary primer (Eight in number HAP-1 to 8) and by using different combinations of anchor and arbitrary primers differentially expressed genes can be identified.

In view of this, in the present work, different developmental stages of carp ovary were chosen for identification of differentially expressed genes by using DDRT. This approach resulted in the identification of THO complex (*thoc*) and Pentraxin (*ptx*) apart from several other known

genes having role in ovarian function. Since, *thoc* and *ptx* were identified from differentiating and differentiated carp ovary for the first time, imparted their crucial role in teleostean ovarian function can be significantly considered for contention. Thus, the present study aimed for comprehensive analysis of *thoc* and *ptx* from development to maturation. Further, role of these genes was also analyzed by localization and tissue as well as reproductive stage–wise expression profiling. Expression pattern was also analyzed at cellular level, germ as well as somatic oogonial and supportive cells. The possible interaction of these two genes, either directly or indirectly, was studied by transient gene silencing using PEI mediated transfection (Höbel and Aigner, 2010, 2013) of *thoc* and *ptx* specific siRNA to understand the impact of their knockdown on various ovary related transcription factors and steroidogenic enzyme genes. Lastly, plausible roles of both genes were analyzed by examining the expression of these correlates in mono–sex feminized fish population from early oogonial proliferation (gonadal determination and differentiation) to ovarian growth.

The findings of these studies compiled into three major chapters for the Ph.D. thesis entitled, "Involvement of *thoc* gene family and *pentraxin* in the ovarian function of *Cyprinus carpio*" as indicated below with a general introduction and a consolidated summary.

Chapter 1. Identification, expression profiling and localization of *thoc* genes in common carp: Influence of *thoc3*–siRNA transient silencing on ovarian development

Chapter 2. Expression analysis of *ptx* during ovarian development and recrudescence, and after siRNA silencing in common carp

Chapter 3. Expression profiling of *thoc3* and *ptx* after estradiol–17β induced feminization of common carp

Each chapter has been provided with a separate bibliography to correlate the findings with earlier reports precisely. All the abbreviations used in the thesis were listed and hence the same has been used without abbreviating again in chapters.

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Chapter 1

Expression profiling and localization of *thoc* genes in common carp: Influence of *thoc3*-siRNA transient silencing on ovarian development

Abstract

In the present study, tho complex (*thoc*) genes were identified in the differentiating ovary of common carp using differential display reverse transcription. To our knowledge, this work is first to characterize the role of *thoc* during ovarian growth and development using expression profiling and transient siRNA silencing. Tissue distribution, ontogeny, reproductive phase profiling of *thoc* revealed spatiotemporal expression in the gonads with high levels from 120 dph. *IS*H and IHC revealed the presence of *thoc3* transcript and protein in stage–III/IV oocytes showing its relevance to vitellogenesis vis–à–vis ovarian growth. Upon cell–level analysis (germ, granulosa, and theca cells), *thoc3*, *thoc5*, and *thoc7* family genes showed differential expression pattern, particularly in granulosa cells demonstrating their contribution to ovarian growth. Transient silencing of *thoc3*–siRNA, *in vivo* and *in vitro* downregulated *ad4bp/sf1*, *amh*, *cyp19a1a*, *foxl2*, *hsd3b*, *hsd11b1*, *hsd20b*, *hsd17b1*, *rspo1*, and *vtg*. Incidentally, *gdf9*, *esr1*, and *igf1* were upregulated while no change was seen in *esr2*, *nanos*, and *vasa*. Reduced levels of serum E_2 after *thoc3* transient silencing indicated differential action on the steroidogenic enzyme, transcription factor, and growth factor genes. Taken together, *thoc3* seems to regulate oogenesis including steroidogenesis, either directly or indirectly.

1. Introduction

Oogenesis is a complex phenomenon wherein various factors/genes differentially expressed during ovarian differentiation, development, growth, and maturation in teleosts. Identification and charaterization of these differentially differentially expressed genes is the first step towards the understanding of ovarian development and growth in teleosts. DDRT-PCR proves to be one of the efficient, convenient and cost-effective methods for such analysis. Unlike microarray-based platforms, DDRT-PCR can be effectively used to detect low expression changes in transcripts, including alternate splice variants (Steinau and Rajeevan, 2009) and for experimentation, the common carp, *C. carpio* were chosen as an animal model in the present study.

C. carpio, a member of Cyprinidae, is a widespread freshwater fish and the International Union for Conservation of Nature declared carp as a vulnerable species in 2008 due to its high demand as food fish. In the Indian sub-tropical conditions, common carp spawn biannually (March and July) and displays gonadal recrudescence with discrete changes in the reproductive cycle. Common carp grows rapidly and reaches 1.0-1.5 kg within a year. Gonadal maturation occurs at the age of six months (~180 dph), and ovary becomes slightly granular and oocytes are easily detectable under a microscope at that stage (Gupta, 1975). Owing to its ecological significance, various genetic and genomic studies have been executed in this species (Kongchum et al., 2011; Ji et al., 2012). Working on this line, in the present study using DDRT-PCR, *thoc* family genes identified from differentiating ovary of 180 dph carp.

In mammals, *thoc* has been well studied which revealed that it is a nuclear component having an indispensable role during transcription elongation of tandemly repeated DNA sequences by the formation of TRanscription EXport (TREX) complex. During protein–

coding gene expression, newly transcribed pre-mRNAs undergo several RNA–processing steps to form mature mRNA. Further, for the subsequent translation process, these-mature mRNA was exported to the cytoplasm. During this exportation, TREX plays a crucial contribution in assembly of co-transcriptional mRNP for mRNA exportation to the cytoplasm (Katahira, 2012; Chang et al., 2013) as well as to recruit other factors for proper formation and export of messenger ribonucleoprotein complexes (Rondón et al., 2010; Luna et al., 2012). The TREX complex in mammals and *Drosophila* is composed of three proteins (THOC1, THOC2, and THOC3) and three additional unique proteins, namely, THOC5/FMIP/fSAP79, THOC6/fSAP35, and THOC7/fSAP24 (Rehwinkel et al., 2004; Masuda et al., 2005). All those THOCs are crucial for normal cell development and differentiation, and their interruption leads to early embryonic lethality and disruption in testis development (Wang et al., 2006, 2009; Mancini et al., 2010). In mammals, *thoc* is essential for embryogenesis (Wang et al., 2006), which intrigues our intention to analyze the physiological relevance of *thoc* during gonadal development and in adulthood in addition to their isolation from differentiating ovary by DDRT-PCR. To our knowledge, there are no reports, implicating a role for *thoc* in ovarian development. As a result, the present work attempts to illustrate the significance of *thoc* family during ovarian development and their interaction with other ovary-related genes. A comprehensive analysis of *thoc* family genes was done to elucidate their functional role by considering tissue distribution, gonad ontogeny, and cell-level analysis. In addition, transient gene silencing of *thoc3* was performed to understand its role in ovarian development.

2. Methods and materials

2.1 Animal Model

Different age groups of common carp reared at Karnataka Veterinary, Animal and Fisheries Sciences University, Bengaluru, India were procured for the present study. Fish from 40 to180 dph (~5-8 cm, 4-7 g) from the same cohort were used for developmental profile analysis while adults (~2-year-old, ~30-40 cm, ~1 kg) were used for tissue distribution, reproductive phase, and cell culture experiments. All the animals were maintained under ambient photothermal conditions during acclimation and experimentation. All animals were fed commercial carp food, *ad libitum* and reared in cement tanks filled with filtered tap water.

2.2 DDRT-PCR

In the current study, DDRT-PCR was basically used to identify genes expressed during carp ovarian differentiation and maturation. These genes may have differential expression during gonad (ovaries and testes) differentiation and maturation. Total RNA was prepared from 180 dph carp ovary (n=3) using Sigma TRI–reagent (Merck, USA; Cat No. T9424) to isolate mRNA through RNeasy Mini Kit (Qiagen, Germany, Cat No: 74104) to perform DDRT-PCR. Quality assessments of purified mRNAs were achieved by using NanoDrop spectrophotometer (ND–2000, NanoDrop Technologies and Wilmington, Delaware, USA).

DDRT-PCR was performed using RNA spectraTM Green1 (Gene Hunter, USA, Cat no: F501) by following the manufacturer's protocol. Three different combinations of anchor primers and arbitrary primer, anchor primers H–T_{11}G, H–T_{11}C, H–T_{11}A, with an arbitrary primer HAP3, respectively, were employed to maximize the effect.

A reverse transcription (20 µl) reaction containing 4 µl of 5x RT buffer, 1.6 µl of dNTP mix, 1 µl (6 µg/µl) of mRNA and H–T_{11}G, H–T_{11}C, H–T_{11}A (2 µM) anchor primers were incubated in a thermocycler at 65°C for 5 min and at 37°C for 10 min, followed by the addition of 1 µl (100 U/µl) MMLV reverse transcriptase and incubated at 75°C for 5 min in a thermocycler. Subsequently, PCR reactions (20 µl) were performed using 2 µl of fluorescent anchor primers: FH–T_{11}G, FH–T_{11}C, FH–T_{11}A (2 µM) with 2 µl of arbitrary primer H–AP3 (2 µM) respectively, 2 µl of 10X PCR buffer, 1.6 µl of dNTP mix, 2 µl of RT mix from the previous step, and 0.2 µl of Taq DNA Polymerase (Qiagen, Germany, Cat no: 201203). The amplification program was as follows, one cycle at 94°C for 30 sec, 40°C for 2 min, and 72°C for 30 sec for 40 cycles followed by 72°C for 10 min. PCR products were electrophoresed on denaturing 6% polyacrylamide gel and scanned using Typhon Imager (GE Healthcare, Sweden) with a 520 nm filter to identify differentially expressed amplicons.

2.2.1 Reamplification of cDNA probes

All amplicons generated in DDRT-PCR were excised from the 6% polyacrylamide gel for sub–cloning and immersed in 100 µl dH_2O for 10 min at room temperature and then boiled for 15 min and spun for 2 min to isolate supernatant. To the supernatant, 10 µl of 3M sodium acetate, 5 µl of glycogen (10 mg/ml) and 450 µl of 100% ethanol were added and stored on –80°C for 30 min, and pelleted by centrifugation at 14000 xg at 4°C for 30 min. After centrifugation, the supernatant was discarded, and the pellet was rinsed with 200 µl of ice–cold ethanol, air dried and then disbanded in 10 µl of dH2O. The extracted DNA was stored at –20°C before being utilized for PCR amplification. The 40 µl PCR reaction included 4 µl of extracted DNA, 4 µl of 10X PCR buffer, 0.3 µl of dNTP mix, 4 µl of arbitrary primer H–AP3 (2 µM), 4 µl of anchor primers H–T_{11}G, H–T_{11}C, H–T_{11}A (2 µM),

and 0.4 µl of Taq DNA Polymerase (Qiagen) and subjected to PCR amplification at 94°C for 30 sec, 40°C for 2 min, and 72°C for 30 sec for 40 cycles followed by 72°C for 10 min. The PCR product was visualized on 1.5% agarose gel, excised and cloned in pTZ57R/T TA cloning vector (Thermo Scientific, USA, Cat no: K1214) using the standard ligation protocol and then sequenced by the Sanger sequencing methodology. Partial cDNA fragments of *thoc* gene family were obtained and confirmed as *thoc1*, *thoc2*, *thoc3*, *thoc5*, *thoc6*, and *thoc7* by NCBI–BLAST. Incidentally, these fragments were also later obtained from transcriptome analysis which has been reported in supplementary data, accession number GSE112157 (Anitha et al., 2019).

2.3 RACE

RACE (5' and 3') strategy was used to obtain the full–length cDNA of all *thoc* family genes using the gene–specific primers designed based on the nucleotide sequence of partial cDNA fragments isolated using DDRT. For this, SMARTer™ RACE cDNA amplification kit (Clontech, USA, Cat. No. 634858) was used for preparing 5' and 3' cDNA templates following the manufacturer's protocol. Subsequently, 5P, 5N, 3P and 3N primers (Table 1) and universal anchor primer A mix and nested universal primer were used in touchdown PCR using Advantage 2 PCR kit (Clontech, USA, Cat. No. 639206) by following manufacturer's thermal cycling conditions (Clontech) to obtain the 5' and 3'ends of all *thoc* forms. After touchdown PCR products were electrophoresed on agarose followed by pGEM®–T easy vector (Promega) clonning, sequenced by sanger sequencing, and analyzed using Lasergene software 7.1.0 (DNASTAR, Madison, WI, USA). ORFs of *thoc* gene family were obtained and confirmed as *thoc1*, *thoc2*, *thoc3*, *thoc5*, *thoc6*, and *thoc7* by NCBI–BLAST.

Table 1: List of RACE primers used for cloning thoc gene family

Gene name/ symbol	5′ Primery Primer(5P) (5′–3′)	5′ Nested Primer (3N) (5′–3′)	3′ Universal Primer (5P) (5′–3′)	3′ Nested Primer (3N) (5′–3′)	GenBank Id
thoc1	CTCCCTCCTCCACGTCCATGCCC	GAGACTTGGAGAGTCTCCGGAGG	GGGCATGGACGTGGAGGAGGGAG	1. GGGGAAGGGCCCAGATCGCA 2. GGACGAGATCGCGGCCAAGCTTGG	MK105904
thoc2	GCACAGCATGGTGAAGACGTCCCTGG	1. GTTTCTCCGTTAGGCTCCTGGTGG 2. GGTTTCCCCGCAAGGTCCTGG	GCCCTCAGGAAGGCAGATTGGGAAG	1. GAAGTCGTCTCAGCGTCTGAAGG 2. GGCCCGATCTCTATGCGTTAGCCATG 3. GGCCCGATCTCTATGCGTTAGCCATG 4. CCGCGAGATGAGCCAGGAAGCCAAGC	MK105905
thoc3	CTTTGGTGCTGACCGTGGCCATGC	CGGTCTTTTTCCAGGACGAACACGC	GCATGGCCACGGTCAGCACCAAAG	GACTGGCCTGTGCGGACGCTTAG	MK105906
thoc5	CAGCATCCGAATCACTTTCATCATC	1. GCAGTCCAGGTTGAAGGCTGCTG 2. CCTCGTGCGTCTGGTCTCGGCC 3. CCTGCACCTGCCTCAACCTGACC	GATGATGAAAGTGATTCGGATGCTG	GGCGGGTCTGGTGCATGAGACGG	MK105907
thoc6	CCTCACGGCACCATCCTCCCCACC	1. GTCACCTCCTCCAACTATCAAGCTG 2. GCTGAGTGCTGGGAACGGAGAG	GGTGGGGAGGATGGTGCCGTGAGG	CCCTTCCTCACTCAACACACTGGC	MK105908
thoc7	GGACGTGGAACTGTTTCTTACGG	CTCATGTCTGTCTGGATGTTGC	GCAACATCCAGACAGACATGAG	CCGTAAGAAACAGTTCCACGTCC	MK105909

2.4 Histological analysis

For histology, 180 dph and different reproductive phase ovary of adult carp (~ 2 years old) were fixed in freshly prepared Bouin's fixative (15:5:1, saturated picric acid, formaldehyde, and glacial acetic acid) for 12-14 hours at RT. The tissues were then finally embedded in paraplast after passing through a graded series of ethanol followed by xylene. The tissues were dehydrated using series of ethanol, cleared in xylene then finally embedded in paraplast. This embedded tissue was sectioned of thicknesses 4 µm using a rotatory microtome (Leica Biosystems, Germany, RM2245). The sections were immersed in xylene for dewaxing the sections followed by treatment of retrograde series of ethanol series for rehydrating sections and finally stained with hematoxylin and eosin. After staining, sections were dehydrated again using a graded series of ethanol and clear in xylene and mounted using DPX mountant (Sigma–Aldrich, USA, Cat. No: 44581). Sections were analyzed and photographed using a DM6 B upright microscope equipped with a model no. DFC 4500 camera (Leica Microsystems, Germany) in combination with the Leica Application Suite X.

2.5 qPCR

Total RNA was prepared from 40 to 180 dph gonads for ontogeny analysis while various tissues (brain, muscular tissue, kidney, liver, egg, ovary) from adult carp (~2 years old) for tissue distribution, reproductive phase, and cell culture experiments as per the method described earlier. To eliminate genomic DNA, DNase-I treatment was performed. By using Verso cDNA Kit (Thermo Fisher Scientific, USA, Cat. No: AB1453A) cDNA synthesis was carried out according to the manufacturer's instructions. Real–time PCR using SYBR Green detection method was performed as per the protocol described in Prathibha and Senthilkumaran (2016), and successful RT was confirmed in all samples by performing PCR amplification of 18s rRNA. qPCR primers for 18s rRNA were designed in such a way that at least one of end of primers should covered in junction of two exons,

Table 2: List of primers used for qPCR analysis.

Gene name/symbol	**Forward primer (5′–3′)**	**Reverse primer (5′–3′)**
	qPCR primers of thoc gene family for gene expression analysis	
thoc1	GAATGTGCAATGACCTCCTCCG	GTGTGCTGCAGTCCGAGTG
thoc2	GTTCAGAGGCATCTCCGTG	CGAGGCTGTCGTGTCTCTTC
thoc3	CCTGACAAACGGAAACGGC	GCCGTTTCCGTTTGTCAGG
thoc5	CGAGGAAGACTGCAGGCTC	GCCCAGCTCAGTGAATTCCTG
thoc6	GGATGCTCTGTGGTGGAGG	CCTACTGACATGATCAGGTCC
thoc7	CCAGAGGAAGGGTTCTCAC	GGCCAAAGCATCATACTCTTGG
	qPCR primers for gene expression analysis	
18s rrna	GCTACCACATCCAAGGAAGGCAGC	CGGCTGCTGGCACCAGACTTG
ad4bp/sf1	TCACTATGGCCTGCTCACCTG	CGCTTGTACATGGGGCCGAAC
amh	CCGTGATGAGCAAAGGACACC	CTGCGGATGACACTCCAAGGC
cyp19a1a	GAGCAGGTCATCTGCTGT	GGATGTCCACCTGTCCCT
esr1	TGTCCGGCCACCAATCA	GTGTCTCCTGCTGTGCTTCAT
esr2	TGTCCTGCAACCAACCA	CCTCTGCTGAGACCCCCACGA
foxl2	GCGTCTCACGCTGTCCGG	GCCGGTAGTTGCCCTTCT
gdf9	CCTGACTTGCGTTGAAGACA	CCAGTGGTTTGAGGTCAGGT
hsd11b1	ATCACAGGGTGCGACTCGGGTTTCGGG	CGGCTGAGTGATGTCCACCTGA
hsd17b1	GACATCCTGGTGTGTAATGCAGG	CTGCCTGTGACCAGGATCCGT
hsd20b	GGGTGTGCCATGCTCTTC	CAGCCCTGACCCGTATGA
hsd3b	GGCTTTTCTGTTCATGCCTG	CACGCGTCAGCTCCGGTGCC
igf1	GCTTTTATTTCAACAAGCCCACAG	CAGAAGGAAGTACATTTGAAGAAC
nanos	ACATGATCAGAGGCATGCAG	TCTCTCCGTTGTGTTTGCAG

giving a single cDNA-PCR product and preventing genomic DNA amplification. Specific primers of *thoc* and many other genes cloned from our laboratory are listed in Table 2.

2.6 FCM of primary oogonial cell lines

FCM was performed by adopting the protocol as described by Kise et al. (2012) to segregate germ cells from other cells (TCs and GCs) using 180 dph carp ovary based on light–scattering properties, in other words, based on cell's morphological characteristics. There were no cell surface markers or transgenesis used for FCM. FS and SS are known indicators of cell size and granularity, respectively. Ovary from 180 dph female carp was dissected from 15-20 individuals, pooled and used for subsequent analysis. After washing with 1 x PBS, ovaries were crushed and kept in a solution of 1 ml of 0.5% trypsin (Life Technologies, USA, Cat. No: 15400054) and collagenase IV (Life Technologies, USA, Cat. No: 17104019) in PBS (pH 8.2) containing 5% FBS and 1 mM $CaCl_2$ for 2 hr at 20°C. The enzymatic reaction was stopped by adding 1 ml of L-15 medium (Life Technologies, USA, Cat. No: L4386–1L). Further, cell suspension (approximately $1x10^6$ cells/ml) was filtered through a nylon filter of 40-µm-pore-sized (MERK, USA, Cat. No: 431750) to eradicate non-dissociated cell clusters followed by placed on ice until FCM. Cells were separated by using MoFlo™ XDP (Beckman Coulter, USA) equipped with a 488–nm argon laser. FS and SS signals were amplified to sort the cells based on the granularity and size to obtain germ cells (at A gate) while the other cells at B gate. The sheath pressure was 7.5 psi, and the flow rate did not exceed 2000 cells/sec during sorting. After isolation, germ and other cell populations were processed for total RNA preparation for performing qPCR to validate cell types using marker genes.

2.7 Isolation of common carp ovarian follicular cells

Following FCM analysis, expression of *thoc* gene family was analyzed in follicular adopting isolation protocol as described in Galas et al. (1999). Adult female common carp weighing 1.5-2 kg were anesthetized using MS222; Tricaine (200 ppm) and then sacrificed to dissect ovaries aseptically. They were then rinsed briefly in DEPC–PBS and cleaned off of om connective tissue. Later, the ovarian tissue was transferred into another beaker containing M199 (Sigma-Aldrich; USA) diluted 4:1 with sterile distilled water. A suspension of follicles was obtained by vigorous mixing using a pipette with a large opening.

In the present work besides primary cell culture as discussed in Galas et al. (1999), we additionally tried to isolate follicular cells from other ovarian cell types. For this, single follicles were punctured by gentle tapping. Punctured follicles were then passed through a 25-gauge needle in order to extract pure follicular cells devoid of other ovarian cell population (yolk globule, CA/CV and e.t.c.). Both isolated follicular cell population and other ovarian cell population were washed frequently with PBS and centrifuged at 2000 x g for 5 min before collection. After collection, follicular cell and other cell population were processed immediately for total RNA isolation. qPCR was done to validate follicular cell and other cell population, using theca and granulosa cell steroid marker genes**.**

2.8 *IS*H

For *IS*H, ovary of 180 dph old common carp was fixed at 4% PFA in PBS at a 4°C overnight to perform *IS*H by following the protocol as per the method described in Rajakumar and Senthilkumaran (2014). The PFA–fixed ovary was washed thrice with PBS, 30 min each at 4 °C followed by tissue embedding onto Tissue–Tek cryomold (Sakura, Finetek Europe B.V., The Netherlands) in OCT compound medium (Leica Microsystems) and stored at -80 °C until sectioning. Further, embedded tissue was sectioned at 4 µm thicknesses onto

poly–l–Lysine coated slides with the help of a cryostat (Leica CM1850, Leica Microsystems. Before starting the experiment slides were kept on a hotplate at 42 °C for overnight to complete dried section. DIG–labeled *in vitro* transcription kit (Roche Diagnostics GmbH, Germany, Cat. No: 11175025910) was used for antisense and sense probes preparation (negative control) of *thoc3*, *5*, and *7* respectively by linearization of pGEM®–T Easy–*thoc* plasmids. For linearization, vector–specific restriction enzymes (zero cutters to insert) were used either T7 or SP6 RNA polymerase, based on sequence orientation from pGEM®T–*thoc* cDNA. NBT/BCIP (Roche Sigma, Cat. No: 11681451001) was used for the detection. Sections were analyzed and photographed using a DM6 B upright microscope equipped with a model no. DFC 4500 camera (Leica Microsystems, Germany) in combination with the Leica Application Suite X.

2.9 IHC

To localize Thoc3 protein IHC was carried out in common carp ovary using rabbit polyclonal antibody to human THOC3 (LifeSpan Biosciences, USA, Cat. No. LS–C31568), that showed ~97.2% identical with the C–terminal part of common carp Thoc3. Before starting IHC, polyclonal antibody was confirmed by western blot analysis where it shows Thoc3 protein in carp ovary lysate For IHC, 180 dph old carp ovary first dissected out followed by immediate removal of the peritoneal membrane, then PBS rinse several times to remove blood and finally fixed in 4% PFA in PBS for overnight at 4 °C. On following day, again several PBS washings was done to remove excess PFA before embedding in freezing medium (Leica Microsystems, O.C.T. compound) onto a cryomold (Tissue–Tek, Sakura Finetek Europe B.V., AJ Alphen aan den Rijn, The Netherlands). Embedded tissue was sectioned at 5 µm thickness onto poly–L–Lysine coated slide using a cryostat (Leica CM1850). For IHC, first sections were treated with TBS + 0.025% Triton X–100 and

blocked with 10% normal goat serum (Merck Bangalore Genei) with in TBS+1% BSA for 2 h. A polyclonal antibody of THOC3 (1: 1000) was prepared in TBS+1% BSA and kept for incubation for overnight at 4 °C. A preadsorbed antibody with excess Thoc3 antigen was used to prove the specific binding for detecting Thoc3 protein. In the subsequent day, the sections were rinsed with TBS+0.025% Triton X-100 (TBST) with gentle agitation and further incubated with 0.3% H_2O_2 in TBS to suppress endogenous peroxide activity. After H_2O_2 incubation, slides were incubated with the HRP-conjugated anti-rabbit secondary antibody (1:5000; Merck Bangalore Genei) at room temperature for 2 h. After 2 h incubation, ABC reagent (Vector Laboratories, Burlingame, CA, USA) was added on slides for 30 min. The slides were then washed with TBST and commercially supplied DAB was used as chromogen and H_2O_2 (Vector Laboratories) for color development. After color development, slides were washed with TBST several time and proceded for dehydration using graded series of ethanol, and finally in xylene to mount in DPX mountant. Sections were evaluated using a DM6 B upright microscope equipped with a model no. DFC 4500 camera (Leica Microsystems, Germany) in combination with the Leica Application Suite X.

2.10 *thoc3*-siRNA administration, *in vivo*

Transient silencing using siRNA has been done by using PEI–based method established earlier (Höbel and Aigner, 2010, 2013) and used in teleost (Prathibha and Senthilkumaran, 2016; Rajakumar and Senthilkumaran, 2014; Laldinsangi and Senthilkumaran 2018). Female carp, 180–220 dph (n = 25) used for the experiments were anesthetized using 100 mg/L of MS 222 (Sigma) in ice–cold water following general guidelines of the Institutional Animal Ethics Committee (IAEC). Specific approval from IAEC is not mandatory as the animal model is a food fish. However, specific approval from

the Institutional BioSafety Committee (IBSC) was obtained for using siRNA treatments (IBSC No. BSK–N–2Aug 2018). Three doses of *thoc3*-siRNA complex (Sigma) 1, 5, and 10 ng dissolved in 100 µl of HEPES buffer containing 100 µg of PEI (Sigma, USA, Cat. No: 408727) was injected directly into the ovary, and the same method was followed for sense siRNA (negative control) which was designed especially against *thoc3* sense nucleotide sequence. Subsequently, all the five groups of fishes (control, sense siRNA and 1, 5, and 10 ng treated *thoc3*–siRNA) were kept separately in water tanks in photothermal conditions for a few minutes to recover from anesthesia. All the injected carp was then maintained in different tanks each holding 50 L capacity of water with continuous aeration and replenishment with fresh water. At day 4, the fish were sacrificed, and ovary was dissected out for western blot and qPCR analysis to examine several genes related to ovarian development. During the experiment, no mortality was observed in any of the groups.

2.11 *thoc3*-siRNA treatment, *in vitro*

For *in vitro* transient silencing experiment, adult female carp ovaries (~1–2 year–old) was used for developing primary cell culture by adopting the protocol as described in Galas et al. (1999). The adult female carp was sacrificed and ovaries were removed aseptically. Ovaries were cleaned off of surrounding connective tissue and washed several times with PBS. After washing, the ovaries were transferred to M199, and vigorous pipetting was performed to obtain a single follicles suspension. Single follicle suspension incubated for 30 min at 37°C in 0.2ml of a collagenase–DNase solution (4 mg/ml collagenase IV, 10 mg/ml DNase and 10 mg/ml BSA) in the M199 medium. During this time, the ovarian cells were agitated using a pipette at least 20 times at intervals of 10 min. The dispersed cells were then centrifuged at 1000 rpm for 5 min followed by 3 washes with M199 medium.

The final pellet was resuspended in a known volume of M199 medium. Isolated cells were counted using hemocytometer, and trypan blue staining was done to check cell viability. Cells were then transferred to separate culture plates containing a 25 ml volume of M199 medium supplemented with 10% fetal bovine serum, 0.25% HEPES buffer solution and 1% of 100 antibiotic–antimycotic solutions and cultured for 48 hr at 37^{o}C in a water–saturated atmosphere of 95% air and 5% CO_2 incubator which appeared to be the optimal temperature for satisfactory cell growth (Galas et al. 1999).

For transfection of siRNA, 2 × 10^5 cells were plated in 24–well plates in 500 µl of M199 medium without antibiotics, in order to attain 50–60% confluent at the time of transfection. Sense (as a negative control) and 1, 5, and 10 ng *thoc3*-siRNA oligomer were diluted in 50 µl OPTI–MEM™ I reduced serum medium (Gibco™, Cat. No: 31985062) and mixed gently. Separately, 1 µl of Lipofectamine™ 2000 transfection reagent (Invitrogen, Cat. No: 11668019) were mixed with 50 µl Opti– MEM I Reduced Serum Medium and incubated for 5 min at room temperature. After the incubation, diluted oligomer and the diluted Lipofectamine 2000 were mixed and incubated further for 20 min at room temperature. Finally, primary ovarian cells were treated with oligomer–Lipofectamine 2000 complexes and kept at 37°C in a CO_2 incubator for 96 hours. MISSION® siRNA (Gibco™, Ref:319850–62) was used as a universal negative control siRNA.

2.12 Western blot analysis

From *thoc3*–siRNA–PEI (Sigma) treated ovary, tissue homogenates were prepared in RIPA buffer [having 50 mM Tris HCl (pH 8.0), 150 mM NaCl, 1% NP–40, 0.5% sodium deoxycholate, 0.1% SDS]. The 30 µg of ovary lysate was separated on a 10% SDS-PAGE and transferred on a nitrocellulose membrane (Pall Life sciences, Port Washington, NY, USA). After transfer, the membrane was blocked by using 3% BSA in TBS +1 % BSA for 2

h at RT and then washed thrice in TBST for 15 min each, before incubating with 1:20000 dilution of polyclonal anti–THOC3 antibody in TBS +1 % BSA at 4 °C for overnight. The next day, membranes were probed with HRP conjugated goat anti–rabbit IgG secondary antibody (Merck Bangalore Genei, Bengaluru, India, Cat. No. 114038001A) for 2 h at RT. Following membrane washing thrice with TBST for 15 min, blots were developed using commercial ECL substrate as per the manufacturer's protocol (Promega, Cat. No. W1001). The same protocol was followed for internal control analysis using anti–β–tubulin antibody. Densitometric analysis (arbitrary units) was not done for all the western blot analysis.

2.13 EIA of E_2 and 17α,20β–DP

Serum E_2 and 17α,20β–DP levels were measured in control and post siRNA treated fish by EIA by following the manufacturer's (Cayman) protocol using an estradiol EIA kit (Cayman, USA, Cat. No. 582251). Intra– and inter–assay variations were within limits specified in the manufacturer's protocol. The sensitivity of the detection limit for E_2 was 6.6 pg/ml and for 17α,20β–DP was 15 ng/ml, and the validation of the assay was done by following the method as described earlier by Prathibha and Senthilkumaran (2016).

2.13 Statistical analysis

All statistical analyses were performed using SigmaPlot 14.0 software (Systat Software Inc., Chicago, IL, USA). All the data are expressed as mean ± Standard Error of the Mean (SEM) which passed both homogeneity and normality tests. One–way ANOVA, followed by Student–Newman–Keuls' (SNK) posthoc test was done for statistical evaluation using SigmaPlot 14.0 software (Systat Software Inc., Chicago, IL, USA). A probability of $P \leq 0.05$ was considered statistically significant.

3. Results

3.1 Molecular cloning of different cDNAs of *thoc* gene family from carp ovary

DDRT with three combinations of primer (HPA3 arbitrary primer with A–, G– and C–anchored primers) were tested for the screening of expressed genes using the 180 dph ovary of *C. carpio* (Fig.1). Highly reproducible patterns of cDNAs namely RNA amplicons were obtained. Members of thoc gene family *thoc1*, *thoc2*, *thoc3*, *thoc5*, *thoc6*, and *thoc7* were obtained by DDRT-PCR and RACE. The ORFs of thoc family genes were deduced after aligning the sequences amplified through 5′ and 3′ RACE. The ORF obtained were of ~1.96 kb for *thoc1* encoding ~654 aa with deduced molecular weight of ~71.94 kDa, ~4.7 kb for *thoc2* encoding ~1557 aa with deduced molecular weight of ~177.59 kDa, ~969 bp for *thoc3* encoding ~323 aa with deduced molecular weight of ~36.3 kDa, ~2.2 kb for *thoc5* encoding ~725 aa with deduced molecular weight of ~82.3 kDa, ~972 bp for *thoc6* encoding ~324 aa with deduced molecular weight of ~35.4 kDa and ~609 bp for *thoc7* encoding ~203 aa with deduced molecular weight of ~23.7 kDa. The full–length cDNA fragments obtained were submitted to GenBank, *thoc1* (MK105904), *thoc2* (MK105905), *thoc3* (MK105906), *thoc5* (MK105907), *thoc6* (MK105908), and *thoc7* (MK105909). The phylogenetic tree was constructed by the neighbor–joining method (http://www.phylogeny.fr/simple_phylogeny.cgi) (Fig. 1) which revealed that *C. carpio thoc* have high homology with *Carassius auratus* and *Danio rerio*.

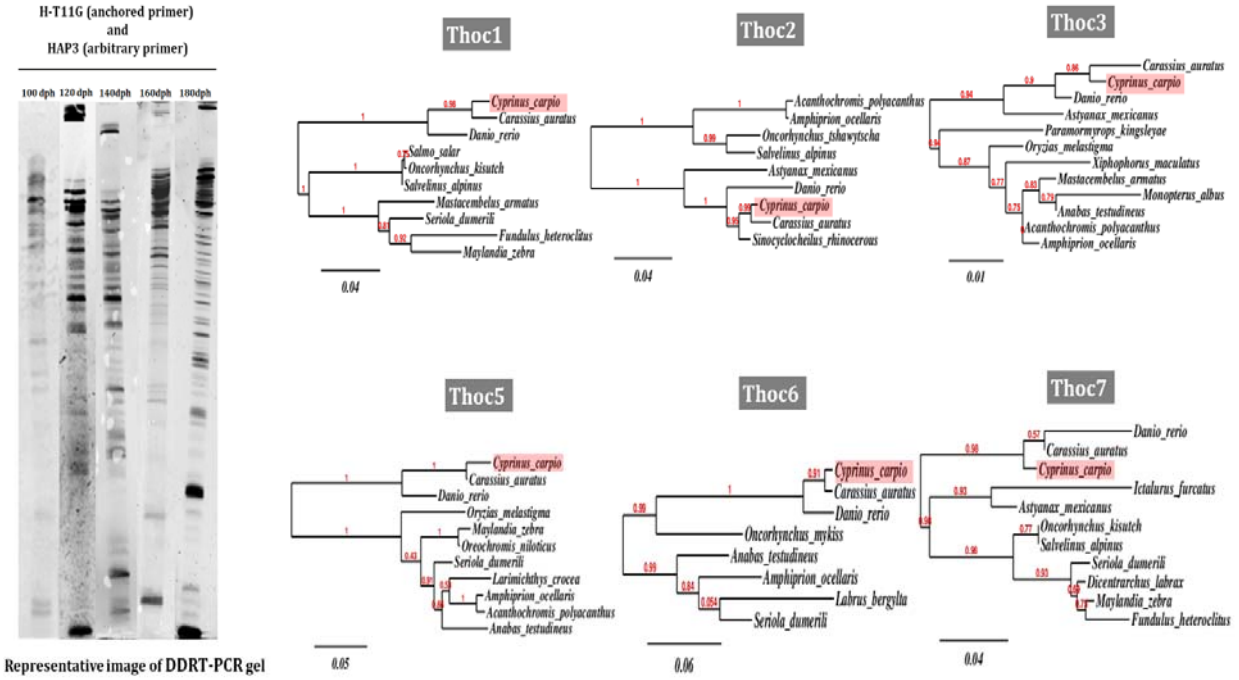

Fig. 1: 6% Urea-PAGE sequencing gel of different *C. carpio* ontogeny ovaries DDRT-PCR with combinations of anchored primer H-T11G and HPA3 arbitrary primer. A phylogenetic tree is showing the evolutionary status of Cyprinus carpio thoc family. The phylogenetic analysis was performed using the neighbour-joining method, and a phylogenetic tree was generated using online phylogeny software.

3.2 Expression of *thoc* gene tissue–wise, ontogeny and ovarian cycle

Tissue distribution of *thoc* gene family revealed exclusive or high expression in ovary (Fig. 2A). Expression analysis of *thoc* gene family in different tissues of adult common carp showed high expression of *thoc3*, *thoc5*, *thoc6*, and *thoc7* in adult carp ovary followed by testis and brain. On the other hand, *thoc2* was found to be high in ovary followed by muscle and kidney, and very low in testis, whereas *thoc1* showed minimal expression in all tissues.

During gonadal differentiation (Fig. 2B) the expression of all *thoc* family genes commenced from 120 dph, of which, much higher expression of *thoc5* was evident. Expression of *thoc3* was significantly evident from 140 dph onwards till adulthood (Fig. 2A) followed by *thoc5* and *thoc7*, respectively (Fig. 2B).

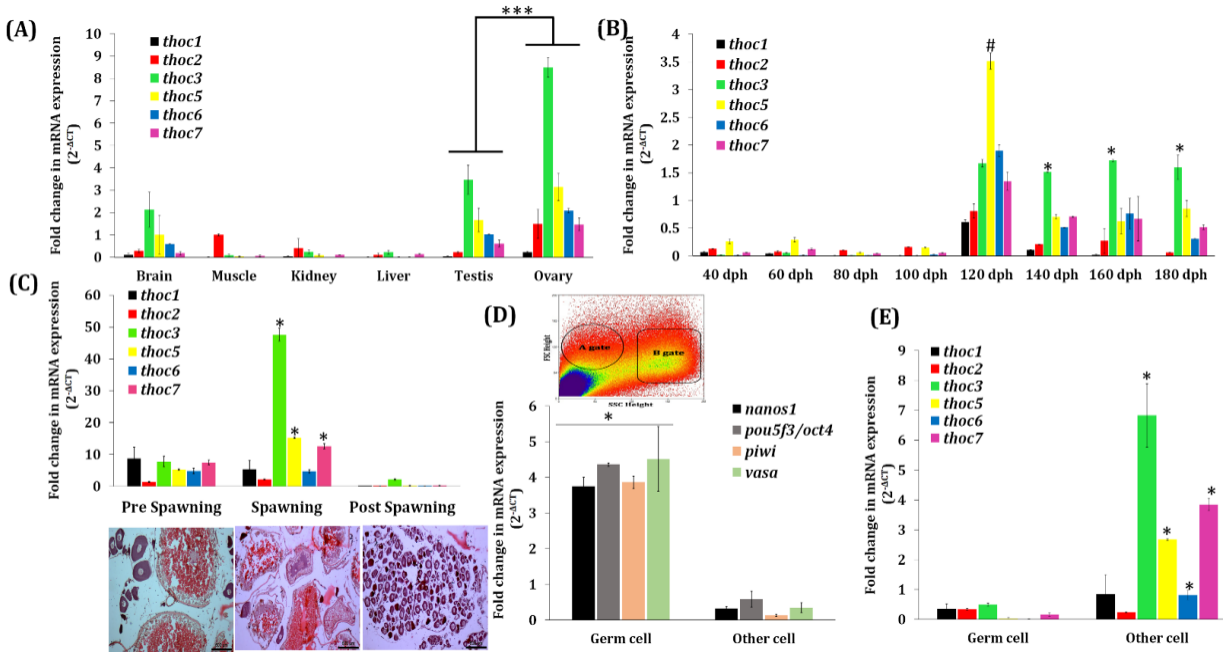

Fig. 2: Relative mRNA levels of thoc family in various adult carp tissues (A), during different stages of gonadal development of carp, between 40 and 180 dph (B), during different stage of reproductive phase with hematoxylin and eosin staining of each reproductive phase (C), and in 180 dph ovary sorted cells with germ cell marker genes (D), with relative mRNA expression of thoc (E). All data were expressed as mean ± SEM. Means with different letters differ significantly (P<0.05; ANOVA followed by Student–Newman–Keuls' post hoc test).

qPCR analysis of ovarian reproductive phase revealed significant (P<0.05) high expression of *thoc3*, *thoc5*, and *thoc7* in spawning when compared to prespawning phase especially, *thoc3* (Fig. 2C). The expression of *thoc1*, *thoc2*, and *thoc6* was similar in pre–spawning and spawning phases. On the other hand, there was no expression of *thoc* family genes during the post–spawning phase of common carp ovarian cycle (Fig. 2C). All the reproductive phases were checked by histology. During the prespawning phase, ovary was at stage III (developing) with the presence of yolk vesicle or CA, having primary oocytes. The size of the oocytes increased due to yolk deposition. The cytoplasm became less basophilic and looked pinkish blue rather than dark blue. In spawning phase, ovary was at stage IV/V (maturing or ripening) characterized by the presence of few secondary

oocytes, but dominated by tertiary oocytes which were characterized by differentiated TCs and GCs from the follicular layer, with predomintely presence of full–grown immature oocytes. Finally, in the post–spawning phase, ovary was at stage VI or I (resting phase or immature) having mainly the primary oocytes.

3.3 Expression of a *thoc* gene family isolated a follicular layer/cells

The expression pattern of *thoc* was checked in germ versus other cells after performing FCM. The germ cell population showed higher expression of *nanos1*, *pou5f3*/*oct4*, *piwi*, and *vasa*, when compared with the population of other cells, hence authenticating the method of segregation using FCM. The expression of *thoc3* was significantly higher in other cells followed by *thoc5*, *thoc7*, and *thoc6* as compared to their counterparts in germ cells.

After examining the expression of *thoc*, an attempt was made to inspect their expression in the ovarian follicular layer using qPCR analysis (Fig. 3A). To validate the isolation, qPCR was performed with specific steroidogenic enzyme gene markers, which are *hsd3b* and *hsd17b1* for TCs, *hsd20b* and *cyp19a1a* for GCs respectively and *gdf9*, and *amh* which are ovarian growth factors. High expression of *hsd3b*, *hsd17b1*, *hsd20b*, and *cyp19a1a* were observed in the follicular layer (Fig. 3B) which confirms the presence of only TCs and GCs. Significantly higher expression of *gdf9* and *amh* was also observed in the follicular layer (Fig. 3C). By contrast, other cell populations consisting of yolk globules, CA/CV and oogonial cells show lower expression of *hsd3b*, *hsd17b1*, *hsd20b*, and *cyp19a1a*.

Among *thoc* family genes, significantly elevated expression of *thoc3*, *thoc5,* and *thoc7* were seen in the follicular layer, while *thoc1*, *thoc2*, and *thoc6* were found to have a similar expression pattern (Fig. 3D).

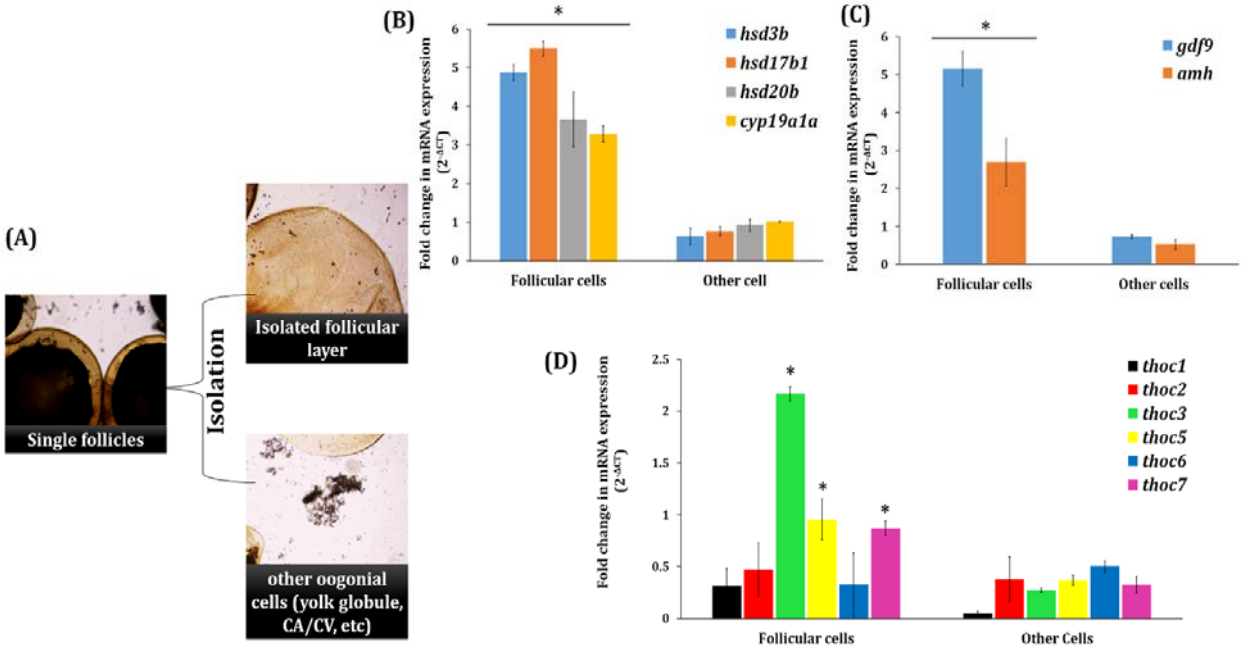

Fig. 3: Isolation of follicular layer from ovary of adult spawning carp (A). Relative mRNA levels of steroidogenic genes in isolated follicular layer cells vs other ovarian cell population (B), ovarian differentiation growth factor genes *gdf9*, and *amh*, in isolated follicular layer (C), and expression of *thoc* family in isolated follicular layer (D). All data were expressed as mean ± SEM. Means with different letters differ significantly ($P<0.05$; ANOVA followed by Student–Newman–Keuls' post hoc test).

3.3 Localization of mRNAs of *thoc3*, *thoc5*, and *thoc7* in carp ovary

Among *thoc* family genes, *thoc3*, *thoc5*, and *thoc7* that showed significantly elevated expression were chosen for localization (Fig. 4A-I). *IS*H revealed the presence of *thoc3*, *thoc5*, and *thoc7* transcripts in stage III/IV vitellogenic oocytes (Fig. 4A-B, D-E, and G-H), which was confirmed using a sense probe (Fig. 4C, F, and I) showing no signal. All three forms of *thoc* expressed on the membrane of CA/CV flattened vesicles (sacs) packed into a continuous layer just under the membrane of III/IV stages of developing oocytes. Incidentally, the transcript signals also extended into the alveoli sacs. Histology sections of ovary stained using haematoxylin and eosin were shown to confirm the stage of oocytes and regions of localization (Fig. 4J-L).

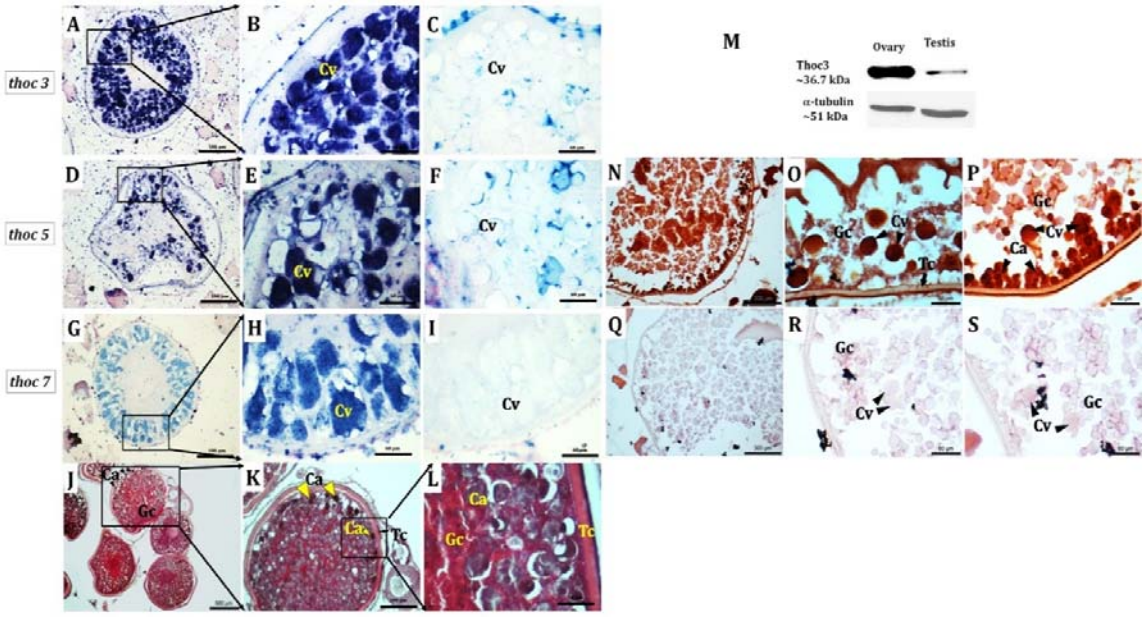

Fig. 4: Localization of mRNAs anti–sense of *thoc3* (A-B), *thoc5* (D-E) and *thoc7* (G-H) and sense of *thoc3* (C), *thoc5* (F), and *thoc7* (I) in the 180~200 dph carp ovary by ISH. Hematoxylin and eosin staining of immature carp ovary (J-L). Western blot analysis is demonstrating the antibody specificity of Thoc3 (M). Localization of Thoc3 in the immature carp ovary by IHC with anti-THOC3 (N-P) and Negative control (Q-S). Arrowhead indicates in ovary sections showing the presence or absence of transcript localization/immunoreactivity. The square box indicates the area for further magnification. Gc: granulosa cell, Tc: theca cell, Ca: cortical alveoli, Cv: cortical vacuole, (Scale bars were indicated inside each figure sub–set).

Based on the results mentioned above, it is possible to assume that *thoc3* might have an essential role in ovarian growth compared to *thoc5* and *thoc7* and hence, it was chosen for further studies.

Western blot analysis was used to validate THOC3 antibody which showed a sharp band of ~36.7 kDa corresponding to Thoc3 (Fig. 4M) in ovary. Further, IHC showed localization of Thoc3 on the membrane of CA/CV along with the theca layer of developing oocyte (Fig. 4N-P) indicated localization gradually reached towards follicular layer with oocyte growth and also validating the localization of *thoc3* transcript. Immunoreactivity was not

evident in preadsorbed of excess antigen THOC3 antibody control (Fig. 4Q–S), confirming the antibody specificity.

3.4 Silencing of *thoc3* in carp ovarian follicle *in vivo* at mRNA and protein levels

Upon transient silencing of *thoc3*-siRNA, the expression levels of *thoc3* reduced significantly ($P<0.001$) to ~39% at a 1ng concentration of *thoc3*-siRNA and ~99% at 10ng concentration after 96 hr in comparison with control (Fig. 5A). Since the transient silencing of *thoc3*-siRNA was performed for a short period of 4 days, no phenotypic changes were evident. Typically, phenotypic changes cannot be imparted in the transient silencing experiment using siRNA (Laldinsangi and Senthilkumaran 2018). The protein levels of Thoc3 also reduced like that of *thoc3* transcripts while no change was evident in control, α–tubulin (Fig. 5B).

Since 10ng *thoc3*-siRNA showed nearly 100% transient knockdown of *thoc3* transcript in ovary when compared to control and hence chosen for the investigation of other genes involved in the ovarian development and growth.

The expression of steroidogenic enzyme genes *cyp19a1a*, *hsd3b*, *hsd11b1*, *hsd17b1*, and *hsd20b* were downregulated ($P<0.05$) by ~71%, ~75%, ~80%, ~33%, and ~43%, respectively when compared to control. The expression of transcription factors which are crucial for ovarian development and maturation, namely, *ad4bp/sf–1*, *amh*, *foxl2*, *rspo1*, and *vtg* reduced significantly ($P<0.05$) by ~70%, ~82%, ~73%, ~77% and ~62% respectively, compared with their expression in control. On the other hand, expression of *esr1*, *gdf9*, and *igf1* were elevated ($P<0.05$) by ~97%, ~75%, and ~70%, and respectively when compared to control (Fig. 5C). There were no significant changes in *esr2*, *nanos*, and *vasa* in comparison with the control (Fig. 5C).

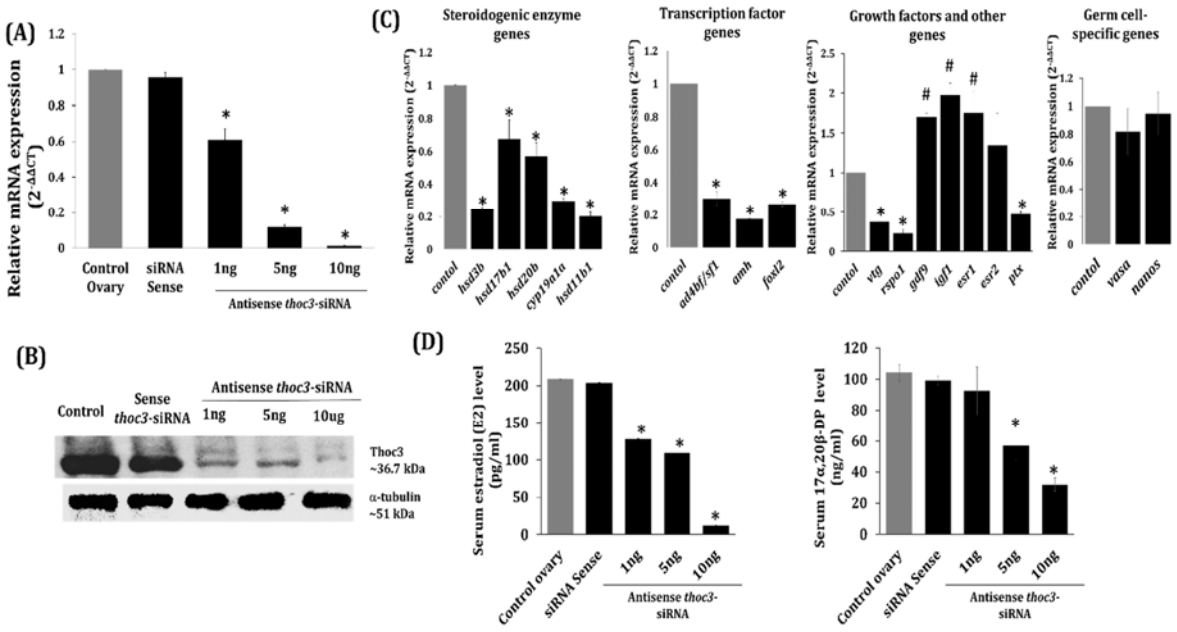

Fig. 5: Relative mRNA levels of the *thoc* family after *thoc3*-siRNA knockdown after 96h *in vivo* (A), Western blot analysis of Thoc3 protein after *thoc3*-siRNA knockdown after 96h *in vivo* (B). Relative mRNA levels of steroidogenic enzymes such as *hsd3b*, *hsd17b1*, *hsd20b*, *hsd11b1*, *cyp19a1a*, transcription factor *ad4bp/sf1*, *amh*, *foxl2*, growth and other factors *vtg, rspo1*, *gdf9*, *igf1*, *esr1/2* in the control and 10ng *thoc3-siRNA* knockdown ovary, *in vivo* (C). Relative serum levels of E_2 and 17α,20β–DP in control and after *thoc3*-siRNA knockdown in carp (D). (*, P<0.05; one-way ANOVA on ranks followed by SNK test). All data were expressed as mean ± SEM. Means with different letters differ significantly (P<0.05; ANOVA followed by Student-Newman-Keuls' post hoc test).

3.5 Change in the E_2 and 17α,20β–DP levels of steroid hormones, after *thoc3-siRNA* in Carp, *in vivo*

Levels of serum E_2 and 17α,20β–DP (Fig. 5D) decreased significantly (P<0.05) after *thoc3-siRNA* treatment when compared to the control. In the case of control group total serum E_2 level calculated was 209.04 pg/ml which was reduced to 128.02 pg/ml, 108.9 pg/ml, and 12.03pg/ml serum E_2 levels and serum 17α,20β–DP level calculated was 104.2 ng/ml which was reduced to 92.5 ng/ml, 57 ng/ml, 31.9 ng/ml in 1ng, 5ng, and 10ng *thoc3-siRNA* treated groups, respectively.

3.6 Silencing of *thoc3* in primary culture of carp ovarian follicle, *in vitro* at mRNA level

The impact of *thoc3*-siRNA knockdown in *C. carpio* primary ovarian culture was evaluated (Fig. 6A–C). The expression of thoc3 transcripts reduced ($P < 0.05$) by ~60% and ~84% for 5 and 10 ng *thoc3*-siRNA *(*Fig. 6A). The transfection efficiency was confirmed with universal siRNA (Fig. 6B).

The transcript expression of steroidogenic enzyme marker genes *hsd3b*, *hsd17b1*, *hsd20b cyp19a1a*, and *hsd11b1* ($P<0.05$) reduced by ~83%, ~76%, ~77%, ~84%, ~60% respectively in 10 ng *thoc3*-siRNA *treated* group as compared to control (Fig. 6C). Furthermore, transcription factors *ad4bp/sf1*, *amh*, *foxl2* were downregulated ($P<0.05$) by ~73%, ~89%, ~90%, respectively, and *rspo1*, *vtg* were downregulated ($P<0.05$) by ~87%, ~73% respectively in 10 ng *thoc3*-siRNA *treated* group as compared to control (Fig. 6C).

In contrast to the mentioned above, the expression of *gdf9* and *igf1* was upregulated ($P<0.05$), while *esr1* and *esr2* expression remain unaffected.

There was no significant change in the expression of genes analyzed after control sense siRNA treatment (Fig. 6A). Since the $2^{-\Delta\Delta Ct}$ calculation was used to check the relative mRNA expression of the steroidogenic enzyme genes, fold change for such genes were not shown. However, fold change has been recorded in Fig. 3B using $2^{-\Delta Ct}$ to denote the differential expression as per cell types.

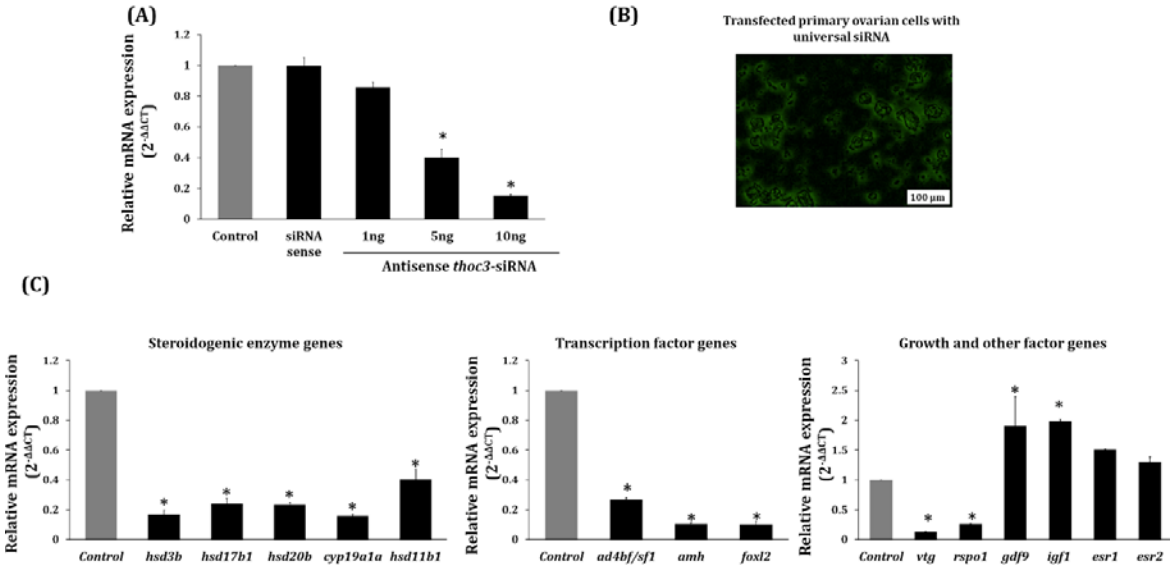

Fig. 6: Relative mRNA levels of the *thoc* family after *thoc3*-siRNA *knockdown* after 96h *in vitro* (A), Representative after control siRNA transfection and fluorescence image of carp ovary primary culture (to check the efficiency) (B). Relative mRNA levels of such as *hsd3b*, *hsd17b1*, *hsd20b*, *hsd11b1*, *cyp19a1a*, transcription factor *ad4bp/sf1*, *amh*, *foxl2*, growth and other factors *vtg*, *rspo1*, *gdf9* in control and 10ng of *thoc3*-siRNA *silencing* of carp ovary primary culture, *in vitro*. (* and #, P<0.05; one–way ANOVA on ranks followed by SNK test). The relative expression ($2^{-\Delta\Delta Ct}$) was normalized with 18S rRNA expression and with gene expression in controls, and the values were calculated using the comparative Ct method. All data were expressed as mean ± s.e.m. (*and # P<0.05; ANOVA followed by SNK test).

4. Discussion

In the present study, full–length cDNAs of *thoc* family (*thoc1, thoc2, thoc3, thoc5, thoc6,* and *thoc7*) were obtained from carp ovary using DDRT-PCR in combination with RACE. The work is first of its kind, signifying the role of thoc family genes during the ovarian function of common carp. Incidentally, earlier reports (Anitha et al., 2019, thesis author 2nd in list) on gonadal transcriptome analysis in common carp yielded cDNAs of *thoc* family identified in this study. In mammals, *thoc* is crucial for mRNA export from the nucleus, yet its precise function in reproduction is largely unknown. Several studies reported the potential role of *thoc* in cell physiology, the viability of early mouse embryo,

postnatal survival, and mouse development in classifying their involvement in cellular differentiation (Wang et al., 2006, 2009; Mancini et al., 2010). However, very little is known about the relevance of thoc function as a consequence of a general genome–wide role. Similarly, it is yet to be discovered whether the *thoc* is equally necessary for reproductive tissues during development. This study provides inquisitive insights to signify role for thoc complex during ovarian development and growth.

Tissue distribution of *thoc* revealed high expression of *thoc3*, *thoc5*, and *thoc7* hierarchically in the ovary, testis, and brain. Differential gene expression of *thoc* in the ovary may indicate a specific role in folliculogenesis and oogenesis as gonadal differentiation has a precise window wherein cascade of gonad–related genes are differentially expressed (McLaren 1988; Jørgensen et al., 2008; Raghuveer et al., 2011; Shi et al., 2013). Developmental profile analysis indicates that thoc expression emanated from 120 dph, wherein 90-120 dph is the critical window of sex differentiation in common carp (Komen et al., 1992). Therefore, the expression of *thoc5* in 120 dph gonad, indicates its role during gonadal development, while *thoc3* expression suggests its role during early and later stages of ovarian growth. Other forms of *thoc*, *thoc1*, *thoc2*, *thoc6*, and *thoc7* also showed elevated expression from 120 dph but subsided later except *thoc6* and *thoc7*. On the whole, these results indicate a plausible role for *thoc3* and *thoc5* in ovarian differentiation and development.

The expression of *thoc3*, *thoc5*, and *thoc7* showed seasonal elevation at the prespawning to reach a maximum at spawning phase indicating their prominent role in ovarian recrudescence and maturation, which might be under the regulatory influence of gonadotropins. Incidentally, FCM analysis also showed high expression of *thoc3*, *thoc5*,

thoc6, and *thoc7* in a group of the follicular cell than the germ cell populations, indicating a significant role for these genes in ovarian steroidogenesis or on other factors that may regulate ovarian growth. Interestingly, *thoc3*, *thoc5*, and *thoc7* transcripts were higher in follicular layer/cell population, composed of TCs and GCs. Both TCs and GCs actively participate in steroid biosynthesis during ovarian growth and maturation (Young and McNeilly 2010). In teleosts, follicles undergo a shift in steroidogenesis during meiotic maturation (Senthilkumaran et al., 2004). Therefore, *thoc3*, *thoc5*, and *thoc7* possibly can be limited to ovarian growth progression as well as maturation.

Overall, expression of *thoc3*, *thoc5*, and *thoc7* signify their presence during ovarian development and especially *thoc3*. Localization study of Thoc3 protein through IHC showed its expression in the follicular layer and membrane of a CA/CV as the oocytes grew to the vitellogenic stage. Due to abundant expression, Thoc3 protein signals even extended into the alveoli sacs. The CA/CV is the first conspicuous cytoplasmic structures, characteristic of teleost oocytes, which can be easily observed under light microscopy (Wallace and Selman, 1981; Bazzoli and Godinho, 1994). In general, CA/CV proliferated as the oocytes grew during maturation and yolk droplets were deposited in the CA/CV region. Parallelly, immunoreactivity with heterologous THOC3 antibody in oocytes detected an intense protein band from an ovarian protein lysate than from testis indicating the presence of Thoc3 expression in the follicular layer as well as CA/CV. Thus, these results support the hypothesis that there is a significant role of *thoc3* in ovarian function by ensuring the ovarian follicular development and growth of CA/CV. It remains to be seen whether the thoc family has any significant role in vitellogenesis.

Based on the above results, it is possible to assume that *thoc3* might have an essential role in ovarian growth compared to other forms of thoc and hence it was chosen for the transient silencing experiments to analyze the functional role of *thoc3* in ovarian steroidogenesis and growth *in vivo*, and *in vitro*. For this a custom-carp *thoc3* siRNA, a synthetic RNA duplex, designed for ovary-targeted *thoc3* transcript degradation specifically but transiently. Control siRNA, sense nucleotide of the *thoc3* transcript, was used to validate our findings. After silencing of *thoc3* mRNA, the expression patterns of *thoc3*, steroidogenic enzyme, transcription, growth, and other factor genes, known to be essential for ovarian development in teleosts, were found to be downregulated by nearly 70–90% in vivo and in vitro both. Upon silencing *thoc3*, *in vivo* and *in vitro*, *cyp19a1a* and *hsd3b* were downregulated. Both steroidogenic enzymes *cyp19a1a* and *hsd3b* are essential for ovarian development and recrudescence (Yoshiura et al., 2003; Wang et al., 2007; Yamaguchi et al., 2007; Senthilkumaran et al., 2009; Raghuveer and Senthilkumaran, 2010; Rasheeda et al., 2010; Raghuveer and Senthilkumaran, 2012). Transcript levels of steroidogenic enzymes *hsd17b1* and *hsd20b* reduced significantly after siRNA treatment in vivo and in vitro. *hsd17b1* modulates gonadogenesis and helps in ovarian development and maturation in catfish (Rajakumar and Senthilkumaran, 2014) while *hsd20b* is known for its pivotal role in teleost oocyte meiotic maturation (Senthilkumaran et al., 2004; Sreenivasulu and Senthilkumaran, 2009) indicating a role for *thoc3* in carp oogenesis. Downregulation of steroidogenic enzyme genes, specifically *cyp19a1a* after *thoc3* siRNA treatment might have been imparted by decreasing the expression of transcriptional regulator, *ad4bp/sf1* and co-activator, *foxl2* (Boerboom et al., 2000; Yoshiura et al., 2003; Senthilkumaran et al., 2004; Wang et al., 2007; Yamaguchi et al., 2007; Rasheeda et al., 2010; Sridevi and Senthilkumaran 2011).

Further, *amh* was also downregulated *in vivo* and *in vitro*, which is a critical hormone during sex differentiation and strictly regulated by *ad4bp/sf1* (Shen et al., 1994). Hence, *thoc3* silencing mediated downregulation of *ad4bp/sf1* attributes for *amh* and *cyp19a1a* downregulation, as the former is a transcriptional regulator of the latter (Yoshiura et al., 2003; Senthilkumaran et al., 2004; Wang et al., 2007; Yamaguchi et al., 2007). Further, knockdown of *thoc3* transcripts downregulates *foxl2* which is a co–regulator of amh and *cyp19a1a* expression (Yoshiura et al., 2003; Jin et al., 2016). Ovarian differentiation factor *rspo1*, an activator of the Wnt/β-catenin signaling pathway, was downregulated *in vivo* and *in vitro*. The *rspo1* activating signaling pathway is essential for ovarian differentiation and maintenance in teleosts (Zhou et al., 2012; Wu et al., 2016) by interacting with transcription factor *foxl2* and *wnt4* to lead ovarian differentiation and development (Biason-Lauber 2012; Pannetier et al., 2016). Downregulation of these transcription factors might have cumulatively altered ovarian steroidogenesis and ultimately ovarian growth. Downregulation of *hsd11b1* was also observed in vivo and in vitro. The changes in *hsd11b1* might alter the precursor androgen availability for E_2 production vis–à–vis follicular development (Michael et al., 1996; Tetsuka et al., 2010). Germline genes, *nanos*, and *vasa* were unaffected after transient siRNA silencing indicating no role for *thoc3* in germ cell proliferation. In contrast to downregulation of steroidogenic enzyme genes, upregulation of *esr1*, *gdf9*, and igf1 *in vivo* and *gdf9*, and *igf1 in vitro* were observed while *esr2* transcript expression *in vivo*, *esr1* and *esr2* expression *in vitro* was unaffected. Interestingly, *esr1/2*, *gdf9*, and *igf1* have a pivotal role in GCs proliferation and differentiation to ensure ovarian follicular development (Drummond et al., 1999; Yang et al., 2004; Otsuka et al., 2011). The precursor protein of egg yolk namely *vtg* was also downregulated in both *in vivo* and *in vitro*. Vtg is a female-specific serum protein, and

synthesized in the liver is under the regulation of E_2 (Meng et al., 2010). Recently, Xue et al. (2018) detected localization of vtg transcripts in oogonia and primary oocytes of turbot and predicted a possibility of autosynthesis of yolk. This remains to be seen in the common carp.

A significant reduction in the serum levels of E_2 after silencing *thoc3* might validate altered transcript levels of steroidogenic enzyme, transcription, growth and other factor genes responsible for E_2 production or regulated by E_2. Hence, *thoc3* silencing suppresses E_2 production, thereby, showing its relevance in ovarian growth and steroidogenesis through the follicular layer. Long term targeted transgenic approach is required for validating this contention. The differential expression pattern is unclear, but an explanation for this can be the downregulation of certain steroidogenic enzyme and transcriptional regulator genes that might have triggered upregulation of *esr1*, *gdf9*, *igf1* to augment steroidogenesis as well as ovarian development.

Taken together, transient silencing and follicular layer analysis revealed a significant role for *thoc3* in regulating ovarian function, more specifically steroidogenesis. Further, spatiotemporal expression of *thoc3* in follicular layer validated potential role in ovarian development and growth. The present work provided the first evidence of interaction of *thoc3* in ovarian steroidogenesis and development in any lower vertebrate using comprehensive transcript expression profile and transient gene silencing analysis.

5. Conclusions

Overall, the present study revealed that *thoc3*, *thoc5*, and *thoc7* transcripts might regulate ovarian growth, development and maturation during the ovarian cycle. Localization of *thoc3* in stage III/IV oocytes implicated a significant role during vitellogenesis. Transient

silencing of *thoc3* imparted differential impact on steroidogenic enzyme, transcription and other factor genes either directly or indirectly. Based on these results, it is possible to implicate a prominent role for *thoc3* in ovarian growth and steroidogenesis of teleosts.

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Chapter 2

Expression analysis of *ptx* during ovarian development and recrudescence, and after siRNA silencing in common carp

Abstract

Pentraxins (PTX), are an evolutionary conserved family containing a pentraxin protein domain. PTX have pattern recognition receptors and known for their acute immunological responses. In the present study, *ptx* cDNA cloned and characterised to examine its expression during ovarian growth and development of common carp through ovary targeted siRNA silencing for the first time. Tissue distribution revealed its exclusive expression in adult ovary. Incremental increase in its expression in pre–spawning phase and reaching to maximum during spawning phase indicated gonadotropin–dependency. Ontogeny study detected *ptx* expression at 120 dph that increased as oocyte development proceed to growth phase. *In situ* hybridization and immunohistochemistry revealed the presence of *ptx* transcript and protein in follicular layer of stage-III/IV oocytes showing its relevance to vitellogenesis vis-à-vis ovarian growth. Transient silencing of was performed to further check the functional significance of *ptx* using follicular primary cell culture, *in vitro* and *in vivo,* in carp through transfection and ovary-targeted injection of *ptx*–siRNA respectively. Upon transient silencing, expression pattern of various genes/factors related to ovarian development such as transcription factors, several steroidogenic enzymes and *esrs* genes were altered. These alteration in expression suggested plausible role for *ptx* in ovarian steroidogenesis either, directly or indirectly which is evident from the changes in the serum E_2 and 17α,20β-DP levels. Taken together, it seems that *ptx* exert an important during ovarian development and/or recrudescence of common carp.

1. Introduction

As described in the chapter 1, oogenesis comprises a series of events where several genes/factors orcheste the process of oocyte development through interaction between oocyte and surrounding somatic cells. In comparison to mammals, the process of oogenesis in teleost offer an extreme diversity due to its vast aquatic environment in-habitate (Wooton, 1984). In recent years, study of teleost oogenesis gained significant scientific attention due to presence of a large number of differentially expressed genes and the dynamics of these in terms of regulation of teleostean oogenesis are not fully understood. In view of this, as discussed in the chapter 1, DDRT approach yielded *ptx* as a differentially expressed in the developing ovary of common carp. This hall leads to identify its significant role in carp oogenesis

PTX also known as pentaxins, is evolutionary conserved protein family, having unique pentagonal plasma protein structure (Du Clos, 2013) with a demonstrated role during acute immunological responses (Gewurz et al., 1995). In mammals, PTX has three principal members namely, CRP, SAP, and PTX3. Both CRP and SAP are short or ‘‘classical” PTXs having characteristic structure of five polypeptides forming a pentagon with 55% amino sequence identity amongst (Woo et al., 1985). Both CRP and SAP are synthesized by liver hepatocytes in response to interleukin (IL)-6 (Garlanda et al., 2005; Casas et al., 2008). On the other hand, PTX3, a long PTX subfamily, expressed and released by dendritic cells, endothelial cells, fibroblasts, and mononuclear phagocytes in response to primary inflammatory signals (Garlanda et al., 2005; Mantovani et al., 2008; Wirestam et al., 2017).

Among PTX members, some reports showed a role for PTX3 in female fertility mainly during cumulus expansion in mammals (Haeger et al., 1992; Salustri et al., 2004; Garlanda et al., 2005; Li et al., 2008). A specific group of granulosa cells, namely cumulus cells, in

mammals form a cumulus cell–oocyte interaction followed by cumulus cell expansion occurs during normal oocyte maturation (Salustri et al., 1992; Chen et al., 2016; Salustri et al., 2018). During this process, expression of several genes/factors including PTX increase drastically upon *gdf9* and bone morphogenic proteins invoking regulatory imparts (Elvin et al., 1999; Yan et al., 2001; Varani et al., 2002). Furthermore, PTX plays an important role in cumulus cell-oocyte interaction as *Ptx* knockout mice became sub–fertile due to lack of such an event (Varani et al., 2002).

In teleosts, PTX like proteins have been identified in several fish species such as australasian snapper (*Pagrus auratus*), common wolf fish (*Anarhichas lupus*), cod (*Gadus morhua*), halibut (*Hippoglossus hippoglossus*), and rainbow trout (*Oncorhynchus mykiss*) and in some fishes only a single form of PTX has been identified (Jensen et al., 1995; Lund and Olafsen 1999; Cook et al., 2005). There no detailed study on the involvement of this gene in relation to teleostean oogenesis.

Hence, in the present work having identified *ptx* by DDRT (as explained in the chapter 1) in developing ovary at 180 dph, a comprehensive analysis of *ptx* gene was done to elucidate the functional role of this correlate by considering tissue distribution, gonad ontogeny, and cell-level analysis in common carp. Localization of *ptx* mRNA and protein in ovary was done using *IS*H and IHC respectively. In addition, transient gene silencing of *ptx* was performed to understand its influence in ovarian function, more specifically steroidogenesis.

2. Methods and materials

2.1 Animals and sampling

Pocuring of animal, rearing of different age group common carp followed by sampling for ontogeny, tissue distribution and seasonal cycle were done as described earlier in the chapter 1.

2.2 Cloning of *ptx* from carp ovary

Isolation of *ptx* gene by DDRT-PCR followed by 5' and 3'RACE was done to obtain full length *ptx* cDNA as described earlier in the chapter 1.

2.3 Phylogenetic analysis

Multiple sequence alignment of deduced amino acid sequences was performed for *ptx* sequences of different teleost and mammals using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalw2/) as described earlier in the chapter 1.

2.4 Quantative PCR

Total RNA isolation, reverse transcription and qPCR were done as described earlier in the chapter 1. Real-time primers for 18s rRNA and *ptx* was designed as described earlier in the chapter 1. Specific primers of *ptx* and many other genes cloned earlier are listed in Table 1.

Table 1: List of primers used for qPCR analysis.

Gene name/symbol	**Forward primer (5′–3′)**	**Reverse primer (5′–3′)**
18s rrna	GCTACCACATCCAAGGAAGGCAGC	CGGCTGCTGGCACCAGACTTG
ad4bp/sf1	TCACTATGGCCTGCTCACCTG	CGCTTGTACATGGGGCCGAAC
amh	CCGTGATGAGCAAAGGACACC	CTGCGGATGACACTCCAAGGC
cyp19a1a	GAGCAGGTCATCTGCTGT	GGATGTCCACCTGTCCCT
esr1	TGTCCGGCCACCAATCA	GTGTCTCCTGCTGTGCTTCAT
esr2	TGTCCTGCAACCAACCA	CCTCTGCTGAGACCCCCACGA
foxl2	GCGTCTCACGCTGTCCGG	GCCGGTAGTTGCCCTTCT
gdf9	CCTGACTTGCGTTGAAGACA	CCAGTGGTTTGAGGTCAGGT
hsd11b1	ATCACAGGGTGCGACTCGGGTTTCGGG	CGGCTGAGTGATGTCCACCTGA
hsd17b1	GACATCCTGGTGTGTAATGCAGG	CTGCCTGTGACCAGGATCCGT
hsd20b	GGGTGTGCCATGCTCTTC	CAGCCCTGACCCGTATGA
hsd3b	GGCTTTTCTGTTCATGCCTG	CACGCGTCAGCTCCGGTGCC
igf1	GCTTTTATTTCAACAAGCCCACAG	CAGAAGGAAGTACATTTGAAGAAC
nanos	ACATGATCAGAGGCATGCAG	TCTCTCCGTTGTGTTTGCAG
piwi	CATCTGTATCACAAGAGGCTGC	CCTGGAAACACCTGTTTTGGAC
pou5f3/oct4	CCGAAAACCCTCAGGATATGTAC	CACACACGTACTACATCTCTCTCC
rspo1	GAGACGAATAAGCACTGAAG	GTGTGCATTTGTTCATATCACG
vasa	CCATGGTGATCGGGAGCA	GTTCCCACAGCGTCCGGT
vtg	CCTTGTTGCAAACAATGTGG	GTGCCAACAGAAGGAAGAGC
ptx	GGATGAATGGACATCGCAGTA	GGCACCCAGGTAGTTATCAGG
star	TCGTCCGAGCCGAGAACGG	TGCCTCCTCCACTCCACTG
cyp17a1	CCATGGCTCCAGCTCTTTCC	CAGTAAGACCAACATCCTGAGTGC
cyp11a1	TCAACCAAGCGGACCACTGT	TCAGGATGCCGTGCCAACTC
Wnt4	TCCACCGACATGTGTGCATC	ATCATAATTTGTTAACAAAT
fdzR	CGCGCTGCCGAGGATGAGTA	GCTGCTTCACCTCCGCTATG
Rspo1	GAGACGAATAAGCACTGAAG	GTGTGCATTTGTTCATATCACG

2.5 FCM of primary oogonial cell suspension

Segregation of germ cells from other ovarian cells type was done by using flow cytometry as described earlier in the chapter 1.

2.6 Isolation of common carp ovarian follicular cells

Following FCM analysis, isolation of follicular cell layer from mature carp ovary was done as described earlier in the chapter 1.

2.7 ISH

In situ hybridization technique was employed to localize *ptx* transcript, which has been described earlier in the chapter 1.

2.8 Production of anti–carp Ptx

For primary antibody production an antigenic determinant peptide (CNQEPHVPKGNVFDWNTIKYD) based on the deduced amino acid (aa) sequence (aa residue:197-216) of carp was commercially synthesized with KLH conjugation of cysteine *ptx* gene (Sigma-Aldrich, USA; Cat No. PEPTIDE-1E). For the genration of Ptx antibody, female mouse (6 week old, n=2) was used and maintained. Following general guidelines of the Institutional Animal Ethics Committee (UH/IAEC/BSK/2018-I/20), the University of Hyderabad, in each mouse, antigenic peptide mixed in incomplete Freund adjuvants was injected subcutaneously. After 15 days, a subcutaneous injection was injected with a booster dose of an antigenic peptide with an equal volume of complete Freund adjuvants to each mouse. Before the first dose and after 2-3 booster doses, preimmune sera and immune sera respectively were collected by retro-orbital puncture from the mouse eye. After blood collection, the blood sample was kept at 4 °C for overnight and the following day serum was collected by centrifuging the blood sample for 9000 g at 4 °C. The antibody validation and specificity was analyzed by western blotting and IHC. During treatment, mouse was fed commercially available dried pellets, *ad libitum*.

2.9 Western blot

For validating anti-carp Ptx antibody, western blot was done as described earlier in the chapter 1.

2.10 IHC

IHC was carried out to localize Ptx protein in common carp ovary as described earlier in the chapter 1.

2.11 *ptx*–siRNA treatment, *in vitro*

To observe the role of *ptx* in ovarian steroidogeneis, *in vitro* transient silencing experiment was performed on carp follicular primary culture cells as described as earlier in the chapter 1.

2.12 *ptx*–siRNA administration, *in vivo*

Role of *ptx* in ovarian steroidogeneis was done by using *in vivo* transient silencing of *ptx* by ovary–targeted injection in carp which has been described earlier in the chapter 1.

2.13 EIA for E_2 and 17α,20β–DP

Serum levels of E_2 and 17α,20β-DP were measured in the control and after siRNA treatment by EIA as described earlier in the chapter 1.

2.14 Statistical analysis

All statistical analyses were performed using SigmaPlot 14.0 software (Systat Software Inc., Chicago, IL, USA). All the data are expressed as mean ± Standard Error of the Mean (SEM) which passed both homogeneity and normality tests. One–way ANOVA, followed by SNK posthoc test was done for statistical evaluation using SigmaPlot 14.0 software (Systat Software Inc., Chicago, IL, USA). A probability of $P \leq 0.05$ was considered statistically significant.

3. Results

3.1 Molecular cloning of different cDNAs of *thoc* gene family from carp ovary

ORF of ~687 bp was obtained of *ptx* from DDRT followed by 5' and 3' RACE, which was then submitted to GenBank (MN073845). The deduced amino acid sequence possesses characteristic PTX domain. To isolate the paralogs of this gene, genomic DNA PCR was performed with primers designed at highly conserved exon with variable intron, which yield no paralogs. Phylogenetic analysis indicated high homology of Ptx with another teleost encounterparts (Fig.1A-B). In addition, it also has some sequence identity with human CRP and mouse SAP proteins (Fig.1A–B). Full length sequence (~834 bp) of *ptx* was obtained having an ORF of ~687 bp that encodes ~228 aa with deduced molecular weight of ~ 25.43 kDa.

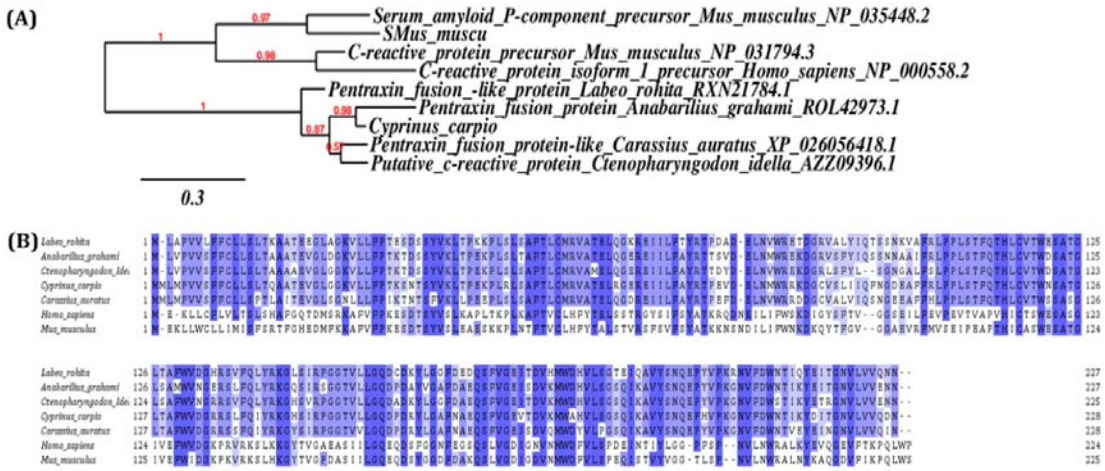

Fig. 1. Multiple alignment of the amino acid sequences of *ptx* from Common carp and other teleosts. The multiple alignment was created using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and high homologous regions are shaded using Jalview 2.8 and important domains are shown as rectangles. The GenBank accession numbers of sequences used are: *Anabarilius graham* (ROL42973.1), *Carassius auratus* (XP_026056418.1), *Ctenopharyngodon idella* (AZZ09396.1), *Labeo rohita* (RXN21784.1), *Mus musculus* (NP_035448.2), *Homo sapiens* (NP_000558.2) and *C. carpio* (submitted to GenBank). Protein–BLAST of Ptx protein of common carp showed conserved with Ptx protein of other teleost group.

3.2 Expression of *ptx:* Tissue distribution, ontogeny, and ovarian cycle

Tissue distribution expression analysis of *ptx* showed predominant expression in ovary followed by minimal or negligible expression in all other tissues (Fig. 2A). Ontogenic expression at various developmental stages from 40 to 180 dph old showed its expression initiated from 120 dph, the critical period of ovary differentiation in carp, to reach a maximum at 160 dph, which became consistent till adulthood (Fig. 2B). qPCR analysis of *ptx* during ovarian reproductive cycle of carp revealed high expression of *ptx* in pre–spawning followed by spawning to reach a nadir at post–spawning phase (Fig. 2C).

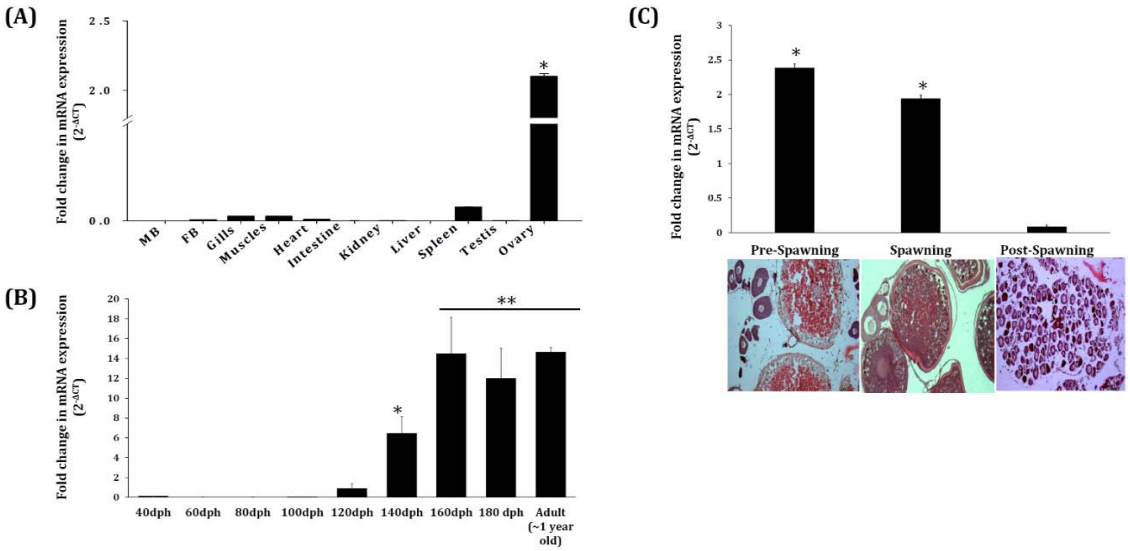

Fig. 2. Relative mRNA levels of *ptx* transcripts. in different tissues of adult common carp (A), during ontogeny (B), during different reproductive phase (C). All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by Student–Newman–Keuls' post hoc test).

All the reproductive phases were confimed using histology, and the oocyte stages were demarked as described by Milton et al. (2018). Presence of more number of stage–III oocytes were evident in prespawning phase, also known as developing phase. At this stage, yolk vesicle or CA were present at the periphery. In addition, lesser number of primary oocytes were seen. In spawning phase, ovary was at stage IV/V (maturing or

ripening), characterized by the presence of few secondary oocytes, yet dominated by tertiary oocytes which were characterized by differentiated TCs and GCs of the follicular layer. The number of full-grown oocytes was found to be high. In the post–spawning phase, ovary was at stage VI or I (resting phase or immature) having mainly the primary oocytes.

3.3 Expression of a *thoc* gene family isolated a follicular layer/cells

Germ cells were isolated by using FCM and expression pattern of *ptx* was observed. At first, FCM isolation was validated by qPCR using germ cell marker genes such as *nanos*, *pou5f3*/*oct4*, *piwi*, and *vasa*. The results showed significant high expression of these genes in isolated germ cell populations when compared with the population of other ovarian cells (Fig.3A). This authenticates the method of cell segregation using FCM. The expression of *ptx* was significantly higher in other ovarian cell population as compared to their counterpart, germ cells (Fig.3B).

Further, to explore other ovarian cells, an attempt was made to isolate follicular layer from the ovarian follicles. This isolation was further validated by performing qPCR using follicular layer specific steroidogenic enzyme marker genes such as *hsd3b*, *hsd17b1*, *hsd20b*, and *cyp19a1a* (Fig. 3C). *hsd3b* and *hsd17b1* are steroidogenic enzyme markers for TCs, while *hsd20b* and *cyp19a1a* are for GCs. In addition, expression of ovarian growth factors *gdf9*, and *amh* were also observed. High expression of *hsd3b*, *hsd17b1*, *hsd20b*, and *cyp19a1a* were observed in the follicular layer (Fig. 3C) which confirms the dominance of follicular layer cells (TCs and GCs). Further, a significantly higher expression of *gdf9* and *amh* was also observed in the follicular layer (Fig. 3D). By contrast, other cell populations consisting of yolk globules, CA/CV and oogonial cells showed lower expression of *hsd3b*,

hsd17b1, *hsd20b*, and *cyp19a1a*. Incidentally, elevated expression of *ptx* was evident in the follicular layer (Fig. 3E).

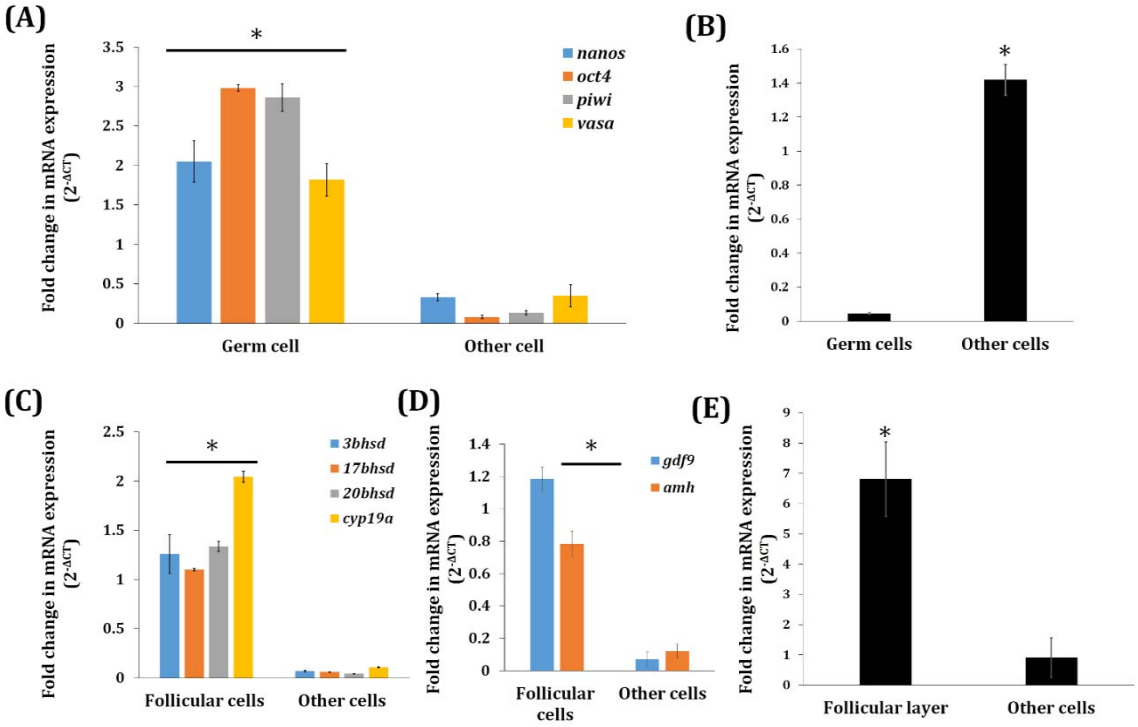

Fig. 3. Relative mRNA levels of germ cell marker gene (A), *ptx* expression in isolated germ cells (B), steroid enzyme marker/other genes (C–D) and *ptx* expression in isolated follicular layer (D). All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by Student-Newman-Keuls' post hoc test).

3.4 Localization of mRNA of *ptx* and Ptx protein

Western blot analysis was used to validate Ptx antibody which showed a sharp band of ~25 kDa (Fig. 4A) exclusively in ovary fractions from adult carp. B- tubulin was shown as equal loading control. *ptx* transcripts (Fig. 4B-C) were localized in the follicular layer of pre vitellogenic and vitellogenic oocytes while no signal was seen in the sense probe (Fig. 4D). IHC analysis also confirmed Ptx protein localization in the follicular layer as that of its transcripts. Immunoreactivity was not evident in the excess Ptx antigen preadsorbed

antibody control (Fig. 4D and E), confirming the specificity. In preabsorbed antibody control, there is no signal (Fig. 4E).

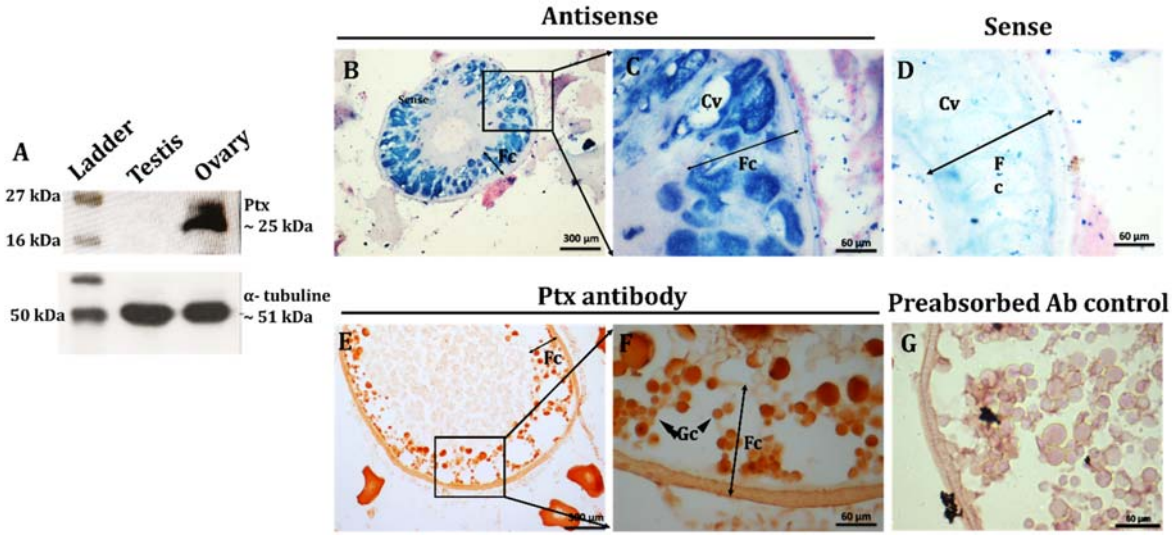

Fig. 3. Western blot analysis for polyclonal Ptx antibody in testis and ovary lysate (A), Localization of mRNAs anti-sense of *ptx* (B-C), and sense of *ptx* (D), in the 180~200 dph carp ovary by ISH. Localization of Thoc3 in the immature carp ovary by IHC with anti–Ptx (E-F) and Negative control (G) in 180–200 dph carp ovary by IHC. Arrowhead indicates in ovary sections showing the presence or absence of transcript localization/immunoreactivity. The square box indicates the area for further magnification. Fc: follicular layer, Gc: granulosa cell, Cv: cortical vacuole, (Scale bars were indicated inside each figure sub-set).

3.5 Silencing of *ptx* in primary culture of the carp ovarian follicle, *in vitro* at mRNA level

The impact of *thoc3*–siRNA knockdown in *C. carpio* primary ovarian culture was evaluated (Fig. 5A–C). There was a reduction in ptx transcript expression ($P < 0.05$) by ~14% upon silencing at 1 ng concentration and finally ~76% at 10 ng *ptx*-siRNA in follicular cell population when compared to control (Fig. 5A) after 48 h. There was no change in *ptx* transcript expression in the population of other ovarian cells after *ptx*–siRNA transfection. Sense siRNA was used as control for transfection and it did not show any changes in expression levels of *ptx* (Fig. 5A). The transfection efficiency of primary follicular layer

culture and other ovarian cell culture were analyzed and confirmed with universal siRNA (Fig. 5B).

The expression of steroidogenic enzyme marker genes *hsd3b*, *hsd17b1*, *hsd20b, cyp19a1a*, and *cyp11a1* ($P < 0.05$) reduced by ~65%, ~51%, ~54%, ~67%, and ~58% respectively at 10 ng *ptx*–siRNA treated group (Fig. 6C) while the expression of *StAR* and *cyp17a1* were ($P < 0.05$) upregulated by ~79% and ~92% respectively as compared to control. Furthermore, transcription factors *ad4bp/sf1* and *foxl2* were downregulated ($P < 0.05$) by ~27% and ~19%, respectively. The expression level of transcripts of *wnt* and Wnt signalling factor, *rspo1* were downregulated ($P < 0.05$) by ~42% and ~30% respectively while their receptor *fdz* expression was upregulated ($P < 0.05$) at 10 ng *ptx*–siRNA treated group as compared to control (Fig. 5B).

There was no significant change in the expression of all above mentioned genes in the population of other ovarian cell types (Fig. 5C).

Since the $2^{-\Delta\Delta Ct}$ calculation was used to check the relative mRNA expression of the steroidogenic enzyme genes, fold change for such genes were not shown. However, fold change has been recorded in Fig. 3B using $2^{-\Delta Ct}$ to denote the differential expression as per cell types.

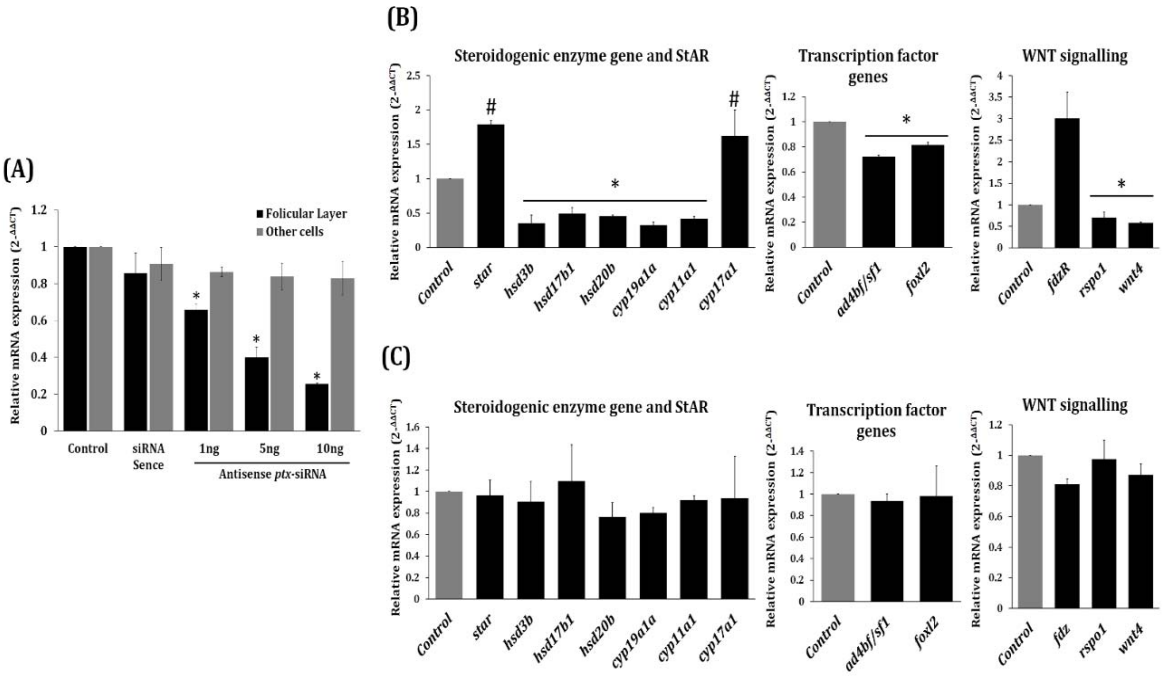

Fig. 5. Relative mRNA levels of *ptx* after transient silencing of *ptx*, *in vitro* (A), expression of steroidogenic enzyme genes, transcription factors and Wnt signalling molecule in carp primary culture of follicular cell layer (B)and in primary cell culture of others ovarian cell type (C). All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by Student–Newman–Keuls' post hoc test).

3.6 Silencing of *thoc3* in carp ovarian follicle *in vivo* at mRNA and protein levels

Upon transient silencing of *ptx*–siRNA, the expression levels of *ptx* reduced significantly ($P < 0.001$) to ~40% at 1 ng concentration of *ptx*–siRNA and ~90% at 10 ng concentration after 96 hr as compared to control (Fig. 6A). As sense probe of *ptx*–siRNA treated group, showed no change in expression, it was used as a positive control of transfection (Fig. 6A). Since the transient silencing of *ptx*–siRNA was performed for a short period of 4 days, no phenotypic changes were evident. Typically, phenotypic changes are not evident in any transient silencing experiment using siRNA for a short duration (Laldinsangi and Senthilkumaran 2018). The protein levels of Ptx also reduced similarly to *ptx* transcripts while no change was evident in control, α–tubulin (Fig. 6B).

The expression of steroidogenic enzyme marker genes *hsd3b*, *hsd17b1*, *hsd20b, cyp19a1a*, and *cyp11a1* ($P < 0.05$) reduced by ~47%, ~46%, ~58%, ~34%, and ~73% respectively at 10 ng *ptx*–siRNA treated group (Fig. 5C) while the expression of *StAR* and *cyp17a1* were ($P < 0.05$) upregulated by ~100% and ~52% respectively as compared to control. Furthermore, transcription factors *ad4bp/sf1* and *foxl2* were downregulated ($P < 0.05$) by ~30% and ~67%, respectively. Ovarian development and maturation specifc transcription factor such as *esr1*, *esr2*, *gdf9*, and *vtg* reduced significantly ($P < 0.05$) by ~35% ~20%, ~60%, and ~81% respectively were downregulated compared with their expression in control. On the other hand, expression of *wnt4* and its activator, *rspo1* were downregulated ($P<0.05$) by ~68% but its receptor expression, *fdz* was upregulated ($P < 0.05$) by ~70% (Fig. 6C).

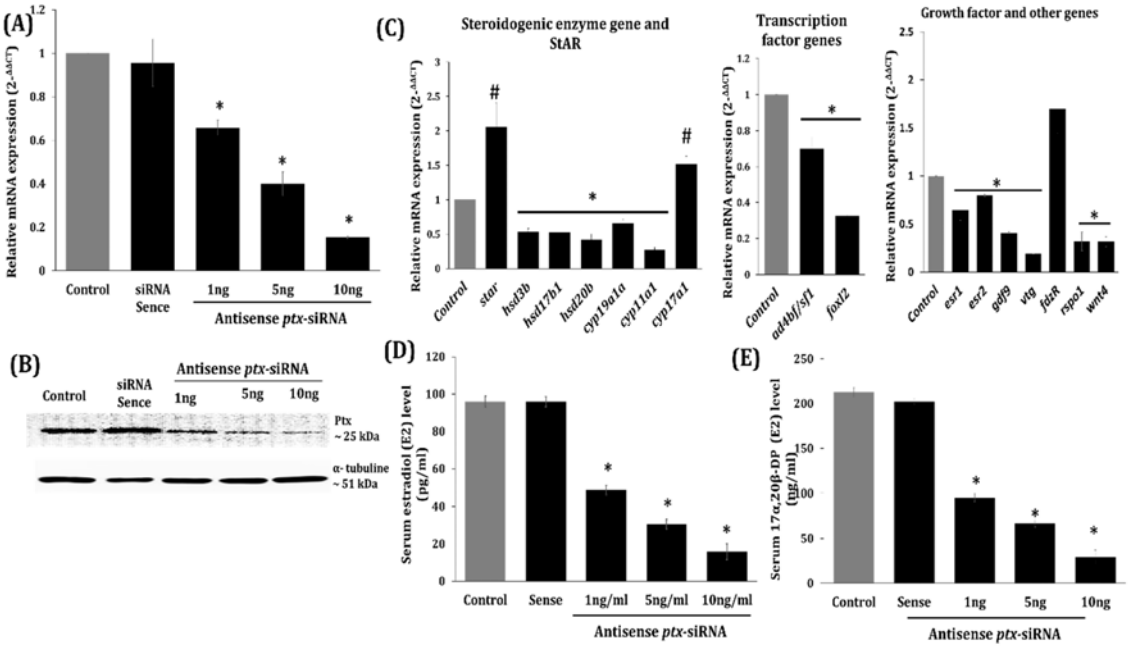

Fig. 6. Relative mRNA levels of *ptx* after transient silencing of *ptx*, *in vivo* (A), expression of steroidogenic enzyme genes, transcription factors and Wnt signalling molecule (B). Estimation of serum E_2 level (D), and 17α,20β–DP (E) after transient silencing of *ptx in vivo*. All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by Student–Newman–Keuls' post hoc test).

3.7 Change in the E_2 and 17α,20β-DP levels of steroid hormones, after *ptx*–siRNA in Carp, *in vivo*

The levels of serum E_2 (Fig. 6 D) and 17α,20β–DP (Fig. 6E) decreased significantly ($P < 0.05$) after *ptx*–siRNA treatment as compared to the control. In the case of control group, serum E_2 level was 96.03 pg/ml which declined to 48.7 pg/ml, 30.62 pg/ml, and 15.81 pg/ml in 1ng, 5ng, and 10 ng *ptx*–siRNA treated groups, respectively. In addition, serum 17α,20β–DP level was 212.85 ng/µl in control which was further reduced to 95.51 ng/µl, 66.6 ng/µl and 29.1 ng/µl in 1 ng, 5 ng, and 10 ng *ptx*–siRNA treated groups, respectively.

4. Discussion

In the present study, full–length cDNA of *ptx* was cloned from carp ovary using DDRT–PCR followed by RACE strategies. PTXs can be induced by acute immunological response due to wide range of expression in a variety of cells such as mononuclear phagocytes, dendritic cells, fibroblasts and endothelial cells in mammals (Garlanda et al., 2005; Mantovani et al., 2008; Wirestam et al., 2017). In addition to that, some studies on its detection and role during female fertility have been elaborated in mammals (Haeger et al., 1992; Salustri et al., 2004; Garlanda et al., 2005; Li et al., 2008). Among PTXs, especially PTX3, coming downstream of GDF9 signalling pathway ensures normal cumulus cell expansion (Salustri et al., 1992; Elvin et al., 1999; Yan et al., 2001; Varani et al., 2002; Chen et al., 2016; Salustri et al., 2018), yet nearly there are no reports on *ptx* in any lower vertebrates.

In the present study, clonning a full length if *ptx* (687 bp) from carp ovary. Tissue distribution and reproductive phase expression analysis of *ptx* showed its elevated expression in adult ovary as well as during ovarian recrudescence, suggesting its possible involvment during ovarian development and growth of common carp. Differential gene

expression of *ptx* during prespawning and spawning phase might be due to difference in hormonal pattern and regulation (Senthilkumaran et al., 2004; Martyniuk et al., 2009) which indicate prominent role for *ptx* in folliculogenesis, where a diverse group of genes/factors were differentially expressed (McLaren, 1988; Jørgensen et al., 2008; Raghuveer et al., 2011; Shi et al., 2013). Expression analysis of *ptx* during ontogeny showed gradual increase in the developing ovary from 120 till 180 dph wherein 75–140 dph is the critical window of ovarian differentiation in common carp (Komen et al., 1992). Therefore, it is possible to implicate a role for *ptx* during gonadal development. Incidentally, FCM analysis also showed high expression of *ptx* in a group of follicular rather than the germ cell populations, indicating a significant role for this gene in ovarian steroidogenesis or on other factors that may regulate ovarian growth. In teleosts, follicular layer is composed of TCs and GCs, active participant during steroid biosynthesis (Young and McNeilly, 2010) and also regulate ovarian growth or oocyte maturation process through activation of different sets of transcription factors by a shift in steroidogenesis (Senthilkumaran et al., 2004). Therefore, possible role of *ptx* cannot be limited to ovarian growth progression, it might extend to maturation as well.

Localization of *ptx* transcript as well as Ptx protein through ISH and IHC showed its expression in the follicular layer of pre– or vitellogenic stage oocytes. Pre– or vitellogenic stages is characteristic stages of secondary growth phase and associated with incorporation and deposition of yolk proteins into specialized membrane bound vesicles i.e., CA (or also known as CV) of growing oocyte follicles (Nagahama, 1983). Further, this membrane bound vesicle were synthesized in the developing oocyte follicle. Various factors such as gonadotropins, *amh*, E_2, regulates the production of CA/CV and their number increased as oocytes grows in size (Lubzens et al., 2010). Thus, present report

indicates plausible role for *ptx* in follicular development. It remains to be seen whether *ptx* has any significant role in vitellogenesis or maturation.

To analyze its function during ovarian development, *ptx* transient silencing experiment was performed in carp follicular primary culture, *in vitro*, and also through ovary targeted injection of siRNA into 180 dph old carp, *in vivo*. For this a custom-carp *ptx* siRNA, a synthetic RNA duplex, designed for ovary–targeted *ptx* transcript degradation specifically but transiently. Control siRNA, sense nucleotide of the *ptx* transcript, was used to validate the findings. After *ptx*-siRNA mediated transient silencing, expression pattern of *ptx*, various ovarian development related transcripts such as steroidogenic enzyme, transcription, growth, and other factor genes, known to be essential for ovarian development in teleosts, were found to be downregulated nearly to 70-90%, *in vitro* and *in vivo* indicating a direct or indirect action of *ptx*. Upon silencing *ptx in vitro* and *in vivo*, there wass downregulation of steroidogenic enzyme genes *cyp19a1a*, *hsd3b*, *hsd17b1*, and *hsd20b*. which are well known for their essential role during ovarian development, recrudescence, and meiotic maturation (Yoshiura et al., 2003; Senthilkumaran et al., 2004; Yamaguchi et al., 2007; Wang et al., 2007; Senthilkumaran et al., 2009; Sreenivasulu and Senthilkumaran, 2009; Raghuveer and Senthilkumaran, 2010; Rasheeda et al., 2010; Raghuveer and Senthilkumaran, 2012; Rajakumar and Senthilkumaran, 2014). These results indicated a pivotal role for *ptx* in carp oogenesis. In teleost, *ad4bp/sf1* and its co–activator *foxl2* regulates the expression of *cyp19a1a* (Yoshiura et al., 2003; Senthilkumaran et al., 2004; Yamaguchi et al., 2007; Wang et al., 2007; Rasheeda et al., 2010; Sridevi and Senthilkumaran, 2011) and downregulation of these factors after *ptx*–siRNA transient silencing showed essential role for *ptx* during ovarian steroidogenesis, more importantly during androgen aromatization to estrogen. Ovarian differentiation

factor *rspo1*, an activator of the Wnt/β–catenin signaling pathway, and *wnt4* were also downregulated *in vitro* and *in vivo.* The *rspo1* has a distinct role in Wnt signaling pathway activation and seems to be essential for ovarian differentiation and maintenance in teleosts (Zhou et al., 2012; Wu et al., 2016) by interacting with transcription factor *foxl2* and *wnt4* to lead ovarian differentiation and development (Biason–Lauber, 2012; Pannetier et al., 2016). Downregulation of these transcription factors might have cumulatively altered ovarian steroidogenesis vis–à–vis ovarian growth.

Further, expression pattern of other factors such as *esr1*, *esr2,* and *gdf9* was also downregulated after transient silencing of *ptx*, *in vivo.* It is known that *esr1/2*, *gdf9*, and *igf1* have a pivotal role in GCs proliferation and differentiation to ensure ovarian follicular development (Drummond et al., 1999; Yang et al., 2004; Otsuka et al., 2011). In addition, expression of vitellogenesis gene i.e, *vtg* also downregulated *in vivo*. Recently, Xue et al. (2018) detected *vtg* transcripts in oogonia and primary oocytes of turbot and predicted a possibility of autosynthesis of yolk. This remains to be seen in the common carp. Taken together *ptx* targets not only steroidognic pathway but also ovarian growth.

In contrast to downregulation in expression pattern of various steroidogenic enzyme and other factors genes, there is a upregulation of expression of *star* and *cyp11a1* after *ptx*–siRNA mediated transient silencing. *star*, a transporter of cholestrol within the mitochondria where *cyp11a1*, a member of the cytochrome P450 superfamily of enzyme localized in the mitochondrial inner membrane, that catalyzes the conversion of cholesterol to pregnenolone, the first rate–limiting step in the synthesis of steroid hormones (Hanukoglu, 1992). In addition, , *fdzR*, a G protein–coupled receptor protein and receptors for Wnt signaling pathway was upregulated (Malbon, 2004). Upregulation

in the expression of *star*, *cyp11a1* and *fdzR* might be a resultant of compensating action for maintaining ovarian steroidogenesis.

A significant reduction in the serum levels of E_2 and 17α,20β–DP after transient silencing of *ptx* might validate altered transcript levels of steroidogenic enzyme, transcription, growth and other factor genes responsible for synthesis and regulation of E_2 and 17α,20β–DP. E_2 have role at varrious levels, predominately during ovarian growth while 17α,20β–DP, is the most potent steroid for inducing final oocyte maturation in several species of fish. Thus, *ptx* might show significant influence on ovarian growth, maturation and steroidogenesis. Long term targeted transgenic approach of *ptx* is required to validate this contention.

5. Conclusions

In the present work, *ptx* was cloning, localization and expression analaysis of *ptx* in common carp ovary during various developemental stages and recrudescence. This is the first detailed report of this gene using a lower vertebrate model to reveal its significant role in oogenesis. Transient gene silencing and follicular layer analysis revealed an important role for *ptx* in regulating ovarian function, more so steroidogenesis. Further, spatio–temporal expression of *ptx* in follicular layer validated potential role in ovarian development and growth. Present work provided evidence of interaction of *ptx* in ovarian development and steroidogenesis in a teleost fish using comprehensive expression profiling and transient gene silencing analysis.

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Chapter 3

Expression profiling of *thoc3* and *ptx* after 17β-estradiol-induced feminization of common carp

Abstract

Sex reversal is one of the promising techniques for aquaculture, mainly in fish to establish mononsex population for growth-based requirement and research. The aim of present work is to investigate the expression of *thoc3* and *ptx* and other genes after inducing feminization.

In order to perform feminization, fingerlings of common carp were exposed to various doses of Cu-NPs which proved unsuccessful as the treatment caused oxidative stress and did not cause sex reversal. However, feminization was successfully done by exposing to exogenous E_2 from 50 dph. In carp, sex determination begins during 29-75 dph while gonad differentiation occurs 75-140 dph. Hence, 50 dph old carp fingerlings were selected for E_2 immersed diet till 50 more days (100 dph) and samples were collected at 50 days intervals of treatment i.e., at 0 (50 dph), 50 (100 dph), and 100 (150 dph) dpt. The expression pattern of various genes after feminization showed upregulation *Star*, *hsd3b*, *hsd17b*, *hsd20b*, and *cyp19a1a* in ovary corresponding to the age of fish. Concomitantly, *thoc3* and *ptx* expression was also upregulated in an age-dependent manner after feminization. These results indicated that *thoc3* and *ptx* are ovary-specific and their expression pattern validated their involvement in oogenesis process in carp.

Part of this thesis published in:


Aquaculture and Fisheries 1 (2016) 15–23

Contents lists available at ScienceDirect

Aquaculture and Fisheries

journal homepage: www.keaipublishing.com/en/journals/aquaculture-and-fisheries/

Original Research Article

Effect of copper nanoparticles exposure in the physiology of the common carp (*Cyprinus carpio*): Biochemical, histological and proteomic approaches

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1. Introduction

Ovarian development is a complex process, regulated by a cascade of genes/factors network to give rise mature gametes for the reproduction. In teleost, a mature ovary has ovarian cavity, germinal epithelium, where primordial germ cells and mitotically active oogonia were reside and stomal compartment which facilitate ovarian growth and steroidogenesis. Unlike the mammals, sex determination is not biased as it controlled by several factors such as genetic, physiological and environmental variables: mainly temperature, pH, and salinity (Devlin and Nagahama, 2002; Strüssmann and Nakamura 2002; Baroiller et al., 2009). Hence, the gender of the fish is not morphologically defined (Devlin and Nagahama, 2002) and shows sexual plasticity. Sexual plasticity is phenomenon where sexes can be manipulated, which can be used effectively in sex control or reversal. Establishment of monosex population is one of the useful approaches in sex control as it is one of the most promising strategies in aquaculture to successfully improve and increase productivity (Wang et al., 2008). These are several methods to manipulate the sex of fish. Use of exogenous steroid hormones is often preferred yet there is other mode by using EDs using pesticide, hormone mimic or NPs of trace elements. Several studies on EDs showed that can interfere with endogenous hormone action and can exert their leading effect to feminization or gonadal disarray (Hayes et al., 2002; Giesy et al., 2003; Körner et al., 2005; Willingham, 2005; Baldigo et al., 2006; Volle et al., 2009; Zoeller et al., 2012; Gore et al., 2013).

There are several studies showing NPs effect on survival and reproduction in teleosts like zebrafish (Yeo and Kang, 2008; Griffitt et al., 2009), perch (Bilberg et al., 2010), brown trout (Scown et al., 2010), *Ceriodaphnia* (Gao et al., 2009), *Daphnia pulex* (Griffitt et al.,

2008). Hence, in order to perform sex reversal, at first, NPs based feminization was attempted. For this, *C. carpio* was exposed to Cu-NPs to observe physiological changes at gene and protein levels. Incidentally, Cu-NPs based approach did not provide a reliable method to induce mono-sex population as it caused oxidative stress (*paper published by thesis author, see below) leading to testicular disarray (Murugananthkumar et al., 2016).

As explained earlier, hormonal or steroid hormone mimic treatments highly preferred to produce physiologically functional mono-sex population. Several studies report that administration of exogenous steroid hormone in immersion baths or by feeds during early ovarian differentiation can cause the feminization (Rougeot et al., 2002; Flynn and Benfey, 2007; Raghuveer and Senthilkumaran, 2008; Arslan et al., 2009). Steroids are crucial for gonadal differentiation in lower vertebrates (Yamamoto, 1969; Bogart, 1987) and it can induce the sex reversal in lower vertebrate, including fish (Nakamura et al., 1998; Baroiller et al., 1999). E_2 has been used in several fish species for feminization study (Park et al., 2004; Wang et al., 2008; Arslan et al., 2009; Raghuveer and Senthilkumaran, 2009). Hence, feminization was induced by feeding E_2 supplemented pellets before ovarian differentiation of carp as the gonad tissue shows plasticity and can respond to exogenous E_2 more efficiently.

Expression of several steroid enzyme genes (*hsd3b*, *hsd17b*, *hsd20b* and *cyp19a1a*) which have major role oogenesis was analyzed to correlate the expression of *thoc3* and *ptx* genes to validate their role during early stages of ovarian differentiation and growth.

2. Materials and methods

2.1 Animals, chemicals and sample collections

Common carp, juvenile (~3 months old) for NPs based trial and fries (40-50 dph old) for hormone exposure experiemts were pocured from Karnataka Veterinary, Animal and Fisheries Sciences University, Bengaluru, India. All the fishes were initially fed with commercial carp food *ad libitum* and reared in glass tanks with countineous areation of water. All other fish related maintanence procedure has been explained in detail in the chapter 1.

I) First NPs trial:

Juvenile common carp were seggregated intwo three groups (20 fish/groups) and exposed to commercially availble Cu-NPs (<50 nm particle size, Sigma, USA, Cat. No.:544868). First and second groups were exposed to 20 µg/L of Cu–NPs for the period of 7 days (i.e. short term exposure). Before re-dosing of Cu-NPs, 80% of water were replaced with fresh water. The selected doses was eco–revalent in most of rivers of India. The thrird group was kept as a control. At the end of the treatments, tissues were dissected out and used for biochemical, histological, and proteomics analysis. For histolog, portion of the tissues were fixed in freshly prepared Bouins fixative (15:5:1, saturated picric acid, formalin, glacial acetic acid) for 12-14 h at room temprature. Tissues from two fishes were pooled to obtained one biological sample and total of five sample were used for all experimentations.

2.2 Histology

Histological analysis of different tissues was performed as per the method described earlier in chapter 1.

2.3 Measurement of oxidative stress

Tissues were homogenized using a micro-pestle in 1.5 ml microcentrifuge tubes which contain equal volumes of 50 mM sodium phosphate buffer (pH 7.4). After homogenization, centrifuged at 10,000 x g for 10 min at 4 °C, and the supernatants were used for the following assay.

a) Catalase (CAT) assay

The measurement of catalase was done as per the method of Beers and Sizer (1952). In brief, H_2O_2 was used as a substrate. The decomposition of H_2O_2 by the catalase enzyme was observed using UV-vis spectrophotometer (UV-1601, Shimadzu, Germany) by measuring the decrease in the absorbance at 240 nm for 5 min. The assay mixture contained 20 µg of protein along with 3% v/v of H_2O_2 in a phosphate buffer (50 mM, pH 7.4) making the final volume of 1 ml. The results were expressed as µM of H_2O_2 consumed/min/mg of protein.

b) Superoxide dismutase (SOD) assay

SOD activity was measured as described by Kostyuk and Potapovich (1989). SOD present in the homogenate blocks the autoxidation of quercetin (Cat No.: Q4951, Sigma). The total reaction mixture contains 1 ml of 0.016 M phosphate buffer, N,N,N',N'–tetramethylethylenediamine buffered with 0.08 mM EDTA. The reaction was *star*ted by the addition of 0.1 ml of 0.015% quercetin solution. For the assay, the supernatant of tissue homogenate, containing 20 µg of protein was added to the mixture to the final

volume of 1 ml and monitored using UV-vis spectrophotometer (Shimadzu) inhibition of auto–oxidation of quercetin by a decrease in the absorbance at 406 nm. Results were expressed as U/mg protein.

c) Glutathione–s–transferase (GST) assay

GST activity was measure by following the protocol of Jakob and Habig (1980). In brief, 20 µg protein was added to the mixture of distilled water, 0.2 M potassium phosphate buffer (pH 7.2), 10 mM L-Glutathione reduced (GSH, Cat. No.: G4251, Sigma) and 0.1 M 1-chloro-2,4-dinitrobenzene (CDNB, Cat. No.: 237329, Sigma). GST catalyzes the conjugation of GSH to CDNB through the thiol group of the glutathione and making CDNB-GSH adduct. The absorbance of the resultant adducts of CDNB (S-2,4-dinitrophenyl glutathione) was measured using UV-vis spectrophotometer (Shimadzu,) at 340 nm. The enzyme activity was calculated based on molar coefficient 9 m/M/cm. Results were expressed as µM of adduct formed/min/mg of protein.

2.4 Two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time of flight mass spectrometric (MALDI-TOF-MS) analysis.

a) Sample preparation

Liver tissue was homogenized using lysis buffer containing 7M urea (Cat. No.: U6504, Sigma), 2M thiourea (Cat. No.: T8656, Sigma), 4% w/v 3–[(3–cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Cat. No.: C9426, Sigma), 40 mM tris-HCl buffer, 1% dithiothreitol (DTT, Cat. No.: D9163, Sigma) and protease inhibitor cocktail (Cat. No.: P8340, Sigma) and centrifuged at 12,000g for 15 min at 4 °C. The supernatant was carefully taken, precipitated using a 2D-cleanup kit (GE

Healthcare, 80648451, United Kingdom) according to manufacturer protocol and final precipitant was dissolved in rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% ampholytes (Cat. No.: P1522, Sigma) of pH 3-10, 30 mM DTT and 0.002% of bromophenol blue, protein concentration was measured by Bradford assay method (Bio–Rad, Cat. #500-0006, CA, USA) as described by Bradford (1976).

b) Isoelectric focusing (IEF) and 2D electrophoresis

The procedures followed for IEF and 2D electrophoresis are based on Laldinsangi et al. (2014). IPG strips (Immobiline Drystrip, 3-10 pH, linear 18 cm; GE Healthcare, 17-1234-01, United Kingdom) were rehydrated with 800 µg of the protein solution for ~20 h under low viscosity mineral oil (BIO-RAD; 163-2129, United States). Isoelectric focusing was performed in Ettan IPGphor3 manifold (Cat. No.: 80-6498-38, GE Healthcare) at 20 °C using the following IEF protocol, 1 h at 50V, ramped 5 h at 500V, 5 h at 500V, ramped 8 h at 10000V, final focusing at 10000V until 70000 Vh. After IEF, the strips were equilibrated twice for 15 min in equilibration buffer (6 M urea, 50 mM tris-Cl (pH 8.8), 30% glycerol and 2% SDS). Subsequently, DTT (1% w/v) and iodoacetamide (4% w/v) were added. Equilibrated strips were placed on 12% polyacrylamide gel and second dimension was performed using Ettan Dalton SDS–PAGE gel apparatus (GE Healthcare). After the electrophoresis, protein spots were visualized in the gel by colloidal Coomassie brilliant blue staining.

c) Image analysis

The gels were scanned using Image Scanner (GE Healthcare), and then analyzed with Image Master 2D Platinum Software 6.0 (GE Healthcare) according to the manufacturer's instructions. All the gels were analyzed for each sample and differentially expressed spots were chosen for further analysis. Based on presence and absence of spots in control and Cu-NPs treated gels, nearly 30 protein spots excised and about 5 differentially distinctly regulated spots of low molecular weight were taken for MALDI-TOF/TOF identification.

d) In–gel digestion, protein identification and database search

MALDI-TOF-MS analysis method described by Shevchenko et al. (1996) with some modifications was adopted using an MALDI-TOF/TOF mass spectrometer (Bruker Autoflex III smartbeam, Bruker Daltonics, Bremen, Germany). Manually excised protein spots were treated with 100 µL of 50% acetonitrile (ACN, Cat. No.: 34967, Sigma) in 25 mM ammonium bicarbonate (NH_4HCO_3, Cat. No.: 09830, Sigma) for 5 times for destaining. Then protein spots were incubated in 10 mM DTT in 25 mM NH_4HCO_3 at 56 ◦C for 1 h followed by 55 mM iodoacetamide in 25 mM NH_4HCO_3 for 45 min at room temperature (25 ± 2 ◦C), washed with 25 mM NH_4HCO_3 and ACN, dried in speed vac (Labconco, SD, USA) and rehydrated in 20 µL of 25 mM NH_4HCO_3 solution containing 12.5 ng/µL trypsin (Promega, Cat. No: V5111, Madison, WI, USA). This sample was incubated on ice for 10 mins and kept for digestion overnight at 37 °C. After completion of digestion, it was centrifuged for 10 min and supernatant was collected in a fresh eppendorf tube. The supernatant treated and vortexed with 50 µL of 1% trifluoroacetic acid (TFA, Cat. No.: 91701, Sigma) and ACN (1:1) for 15 min. After previous treatment the supernatant was pooled, and speed vac was used to dry the supernatant and reconstituted in 5 µL of 1:1

ACN and 1% TFA. 1 µL from the above sample was mixed with 1 µL of freshly prepared α-cyano-4-hydroxycinnamic acid (CHCA, Cat. No.: C8982, Sigma) matrix in 50% ACN and 1% TFA (1:1) and 1 µL was spotted on target plate. Mass data was acquired by flexControl 3.0 (Bruker Daltonics, Bremen, Germany) software using automatic switching between MS and MS/MS modes. Mass range of m/z 800-3500 in the reflectron positive ion mode was accumulated from an average of 2500 laser shots with an acceleration of 19 kV. Fragmentation of precursor ions was achieved using an MS/MS positive mode. Contaminating trypsin and keratin corresponding ions were omitted from the peak lists before database analysis using flex analysis software. By using MASCOT program (http://www.matrixscience.com) using Biotools software (Bruker Daltonics) protein spots identification were performed by database searches (Peptide Mass Fingerprinting and MS/MS). The similarity search for mass values was done with existing digests and sequence information from NCBInr and Swiss-Prot database. The search parameters were as follows: fixed modification of carbamidomethyl, variable modification of oxidation (M), enzyme trypsin, peptide charge of 1^+ and monoisotropic. According to the MASCOT program, the probability of $P<0.05$ was considered statistically significant, and only significant hits were accepted for protein identification.

II) **E_2 exposure expriements**

a) Feed preparation

The feed with different doses of E_2 were prepared one week before *star*ting the experiment. A stock solution of E_2 was dissolved in absolute ethanol and further aliquoted stock solution, to get desired hormonal concentration, which was diluted in 500 ml of 96 % ethanol and mixed homogenously with 1 kg of groundnut oil cake and rice bran in

powder form (in 1:1 (w/w) ratio). The feed was spread on tray, protected from light, for complete alcohol evaporation. After drying the feed was pelleted and stored for feeding during the experiment. During the experiment, the feed was provided *ad libitum* daily to juvenile, four times a day till end of the experiment. Control group were fed only groundnut oil cake and rice bran mixed pellet without any hormone exposure.

b) Exogenous E_2 exposure to common carp

The 200-300 juveniles of common carp were used and maintaind in large glass tank with contineous areation in water. The expriements was performed in two stages. The first stage was feminition or period of hormonal treatment with three diets with incresing level of E_2 i.e. 50, 100 and 150 mg E_2/kg feed over 50 days and one group kept as a control. Each diet was considered as a treatment with three replicates. The second stage (post-feminization) was carried out and fish were fed with commercial feed without hormone. Samples were collected from 0 days treatment (starting date, 50 dph old carp), 50 dpt (end of hormone treatment, 100 dph old carp), 100 dpt (50 days after hormone treatment, 150 dph old carp) respectively from E_2 fed and control groups for expression as well as histological analysis. There is no mortality observed throughout the experiment.

2.5 Quantative PCR

Total RNA was prepared from samples collected during and after E_2 treatments for the expression analysis as per the method described in chapter 1. Specific primers of *thoc3*, *ptx* and many other genes cloned from our laboratory are listed in Table 1.

Table 1: List of primers used for qPCR analysis.

18s rrna	GCTACCACATCCAAGGAAGGCAGC	CGGCTGCTGGCACCAGACTTG
star	TCGTCCGAGCCGAGAACGG	TGCCTCCTCCACTCCACTG
cyp19a1a	GAGCAGGTCATCTGCTGT	GGATGTCCACCTGTCCCT
hsd17b1	GACATCCTGGTGTGTAATGCAGG	CTGCCTGTGACCAGGATCCGT
hsd20b	GGGTGTGCCATGCTCTTC	CAGCCCTGACCCGTATGA
hsd3b	GGCTTTTCTGTTCATGCCTG	CACGCGTCAGCTCCGGTGCC
thoc3	CCTGACAAACGGAAACGGC	GCCGTTTCCGTTTGTCAGG
ptx	GGATGAATGGACATCGCAGTA	GGCACCCAGGTAGTTATCAGG

2.6 Statistical analysis

Data are expressed as mean ± SEM (n = 5). Pair wise comparisons were done by one-way ANOVA followed by Student-Newman-Keuls' test using Sigma Plot 11.0 (Systat software Inc., Chicago, USA) software. The level of significance was $P<0.05$ for all comparisons. A probability of $P<0.05$ was considered as statically significant.

3. Results

3.1 Effect of Cu–NPs on fish body and tissue weight

At the end of the treatment, weight of the whole fish and tissue (kidney, liver and gill) were noted for each group (Table 2). After the treatments, lower dose treated groups showed significant ($P<0.05$) difference in tissue weight and body weight. However, higher dose treated groups showed more significant ($P<0.01$) increase of tissue (kidney, liver and gill) and body weight of whole fish in comparison with control.

Table 2: Effect of Cu-NPs on the body and tissue (Liver, Kidney and Gills) weight of control and treated groups of common carp. Data (n = 10) were expressed as mean ± SEM. (*, P < 0.05; **, P < 0.01; ANOVA followed by Student-Newman–Keuls' post hoc test).

	Weight of fish (gm)	*Tissue weight (gm)*		
		Liver	*Kidney*	*Gills*
Control	8.03 ± 0.297	0.145 ± 0.014	0.142 ± 0.006	0.383 ± 0.028
Lower dose (20 µg/l)	8.672 ± 0.315*	0.159 ± 0.012*	0.160 ± 0.008*	0.482 ± 0.029*
Higher dose (100 µg/l)	10.41 ± 0.786**	0.220 ± 0.024**	0.181 ± 0.010**	0.585 ± 0.039**

3.2 Effect of Cu-NPs on antioxidant enzymes (CAT, SOD and GST) between control and treated groups

Levels of various antioxidant enzymes (CAT, SOD, and GST) were analyzed in kidney, liver and gills of control and treated groups of common carp. Fig 1a represents the change in the activity of catalase between control and Cu-NPs treated groups. The amount of H_2O_2 (substrate) breakdown by CAT directly measured as the units of catalase production. Results exhibited a significant ($P<0.05$; $P<0.01$) increase in the level of substrate utilized in the treated batches (lower and higher dose) of kidney, liver and gill. Similarly, in SOD enzyme assay was done by inhibition of quercetin auto–oxidation. Quercetin oxidizes at pH-10 was shown to be a free radical chain reaction involving superoxide and hence usable for SOD. So, the function of SOD was directly correlated with the degree of inhibition of quercetin oxidation. In the present study, both lower and higher dose treated groups revealed significant ($P<0.05$; $P<0.01$) presence of high function SOD activity in kidney, liver and gill tissues when compared to control group (Fig. 1b). In the case of GST, GST catalyzes the conjugation of GSH to CDNB through the thiol group of the glutathione and making CDNB-GSH adduct and this CDNB–GSH adduct was advantageous to measure the concentration of GST activity. The absorbance of the resultant adducts of CDNB-GSH

adduct showed a significant (P<0.05; P<0.01) elevation in their levels in kidney, liver and gill tissues in treated group (lower and higher dose) when compared to the control (Fig. 1c).

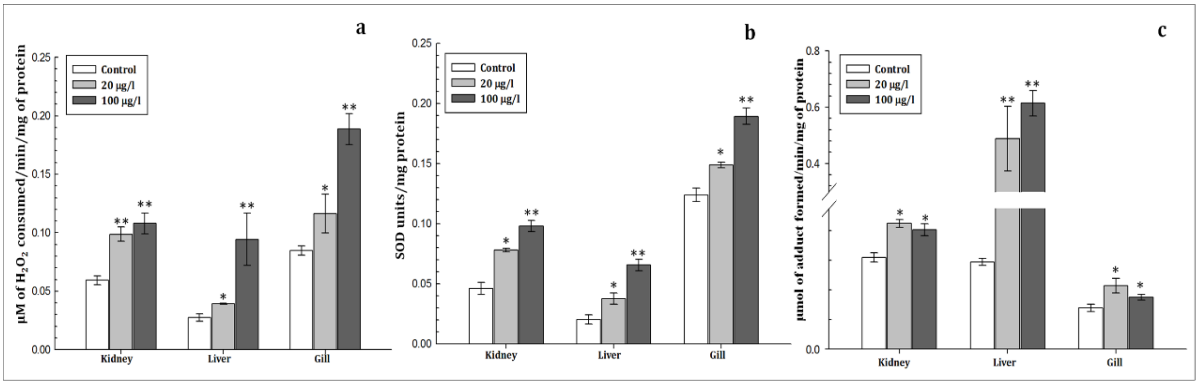

Fig 1: Relative antioxidant enzyme assay in kidney, liver and gill of common carp following the exposure of Cu–NPs for 7 days. (a) CAT enzyme assay, (b) SOD enzyme assay, and (c) GST enzyme assay. Data (n = 10) were expressed as mean ± SEM. (*, P<0.05; ANOVA followed by Student–Newman–Keuls' post hoc test).

3.3 Histological analysis

3.3.1 Kidney

Kidney of control fish showed normal histology with parietal epithelium of Bowman's capsule, glomerulus, proximal and distal tubules (Fig. 2a-b). Lower dose treated groups displayed degeneration of renal tubules, a few necrotic cells in the hematopoietic tissue and presence of sinusoidal space (Fig. 2c-d). Further higher dose groups exhibited a greater number of degenerate tubules and increased in the Bowman's space (Fig. 2e-f).

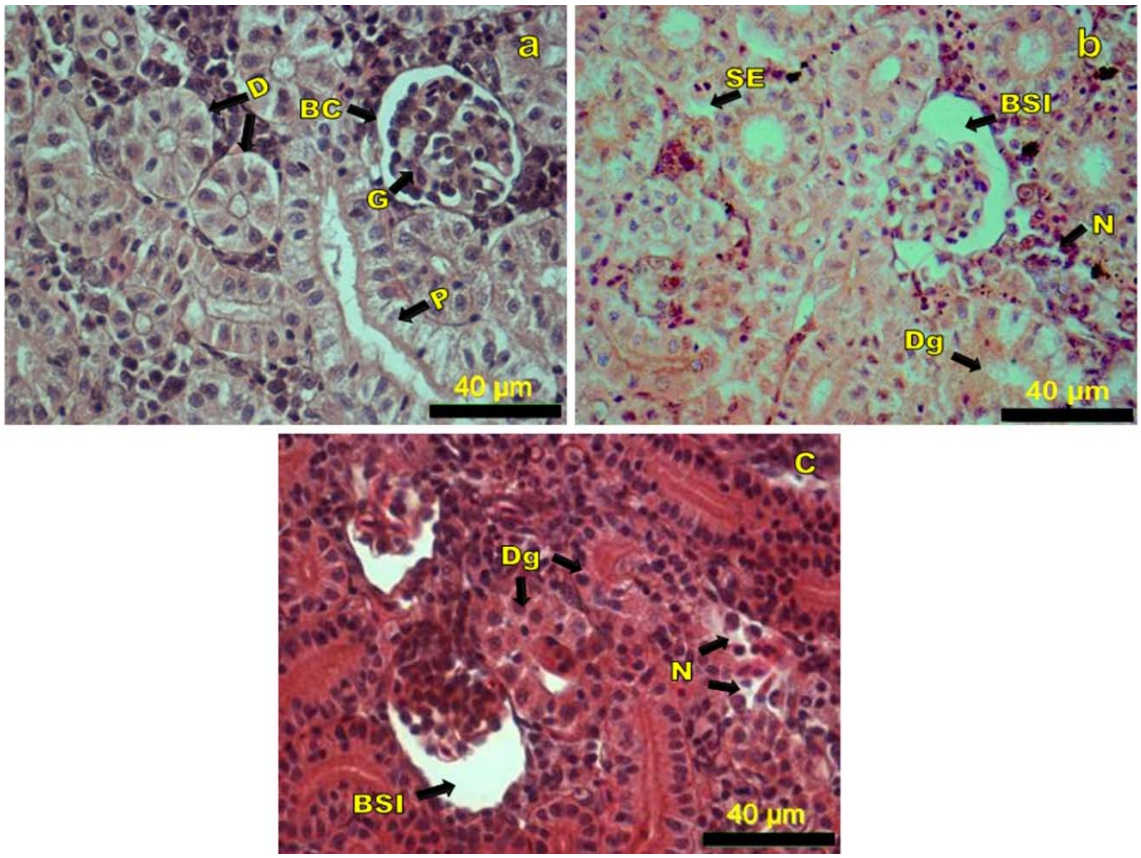

Fig 2: Histological analysis of kidney in common carp following the exposure of Cu-NPs for 7 days. (a-b) control, (c-d) lower dose, (e-f) higher dose. Kidney of control fish showed normal histology with parietal epithelium of Bowman's capsule (BC), glomerulus (G), proximal (P) and distal (D) tubules. Treated group exhibited with the presence of sinusoidal space (SE), a greater number of degenerate tubules (Dg) and increased in the Bowman's space (BSI). All scale bar indicates 40 µm.

3.3.2 Liver

Histological analysis of control liver showed normal hepatocytes with sinusoidal space (Fig. 3a-b). Whereas the lower dose treated groups showed significant changes with increased sinusoidal space, cells with pyknotic nuclei and presence of cytoplasmic vacuoles indicating early stages of necrosis (Fig. 3c-d), the higher dose treatment showed extensive liver damage for example, hepatocytes with pyknotic nucleus and/or cell with dead nucleus, in addition to aggregation of blood cells and damaged blood vessel (Fig. 3e-

f). Since importance of liver is a key organ in the supply, storage, and excretion of Cu pertaining to metabolism in developing fish, we performed proteome analysis only in that tissue.

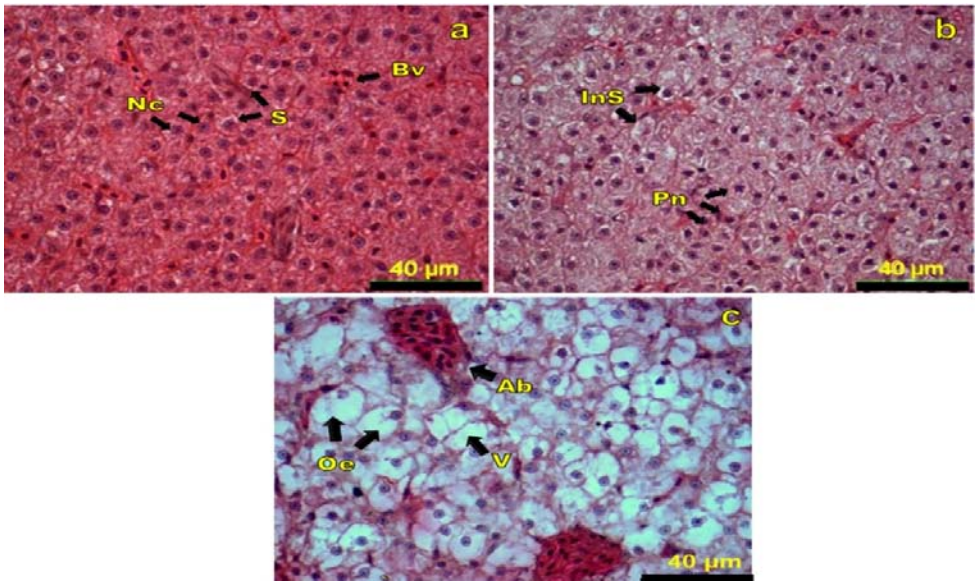

Fig 3: Histological analysis of liver in common carp following the exposure of Cu–NPs for 7 days. (a–b) control, (c–d) lower dose, (e–f) higher dose. The livers of control fish showed normal histology with normal cells (Nc), Normal sinusoid space (S) and normal blood vessel (Bv). Both materials caused similar types of injuries, although these were worse in the equivalent CU–NP treatment by day 10. These injuries include cells Both concentrations caused similar types of Treated groups showed injuries like cells with increased sinusoid space (InS), pyknotic nuclei (Pn), vacuole formation (V), necrosis (N), oedema in the tissue (Oe), and aggregation of blood cell (AB) in the liver. All scale bar indicates 40 µm.

3.3.3 Gills

Gill morphology of control fish displayed normal appearance of primary and secondary lamellae (Fig. 4a-b). Treatment with lower dose of Cu-NPs revealed hyperplasia at the base of the secondary lamellae, oedema of the gill epithelium, lamellar fusion, clubbed tips, occasional aneurism in the secondary lamellae and swollen mucocytes (Fig. 4c-d) and similar changes were observed in fishes exposed to higher dose (Fig. 4e-f).

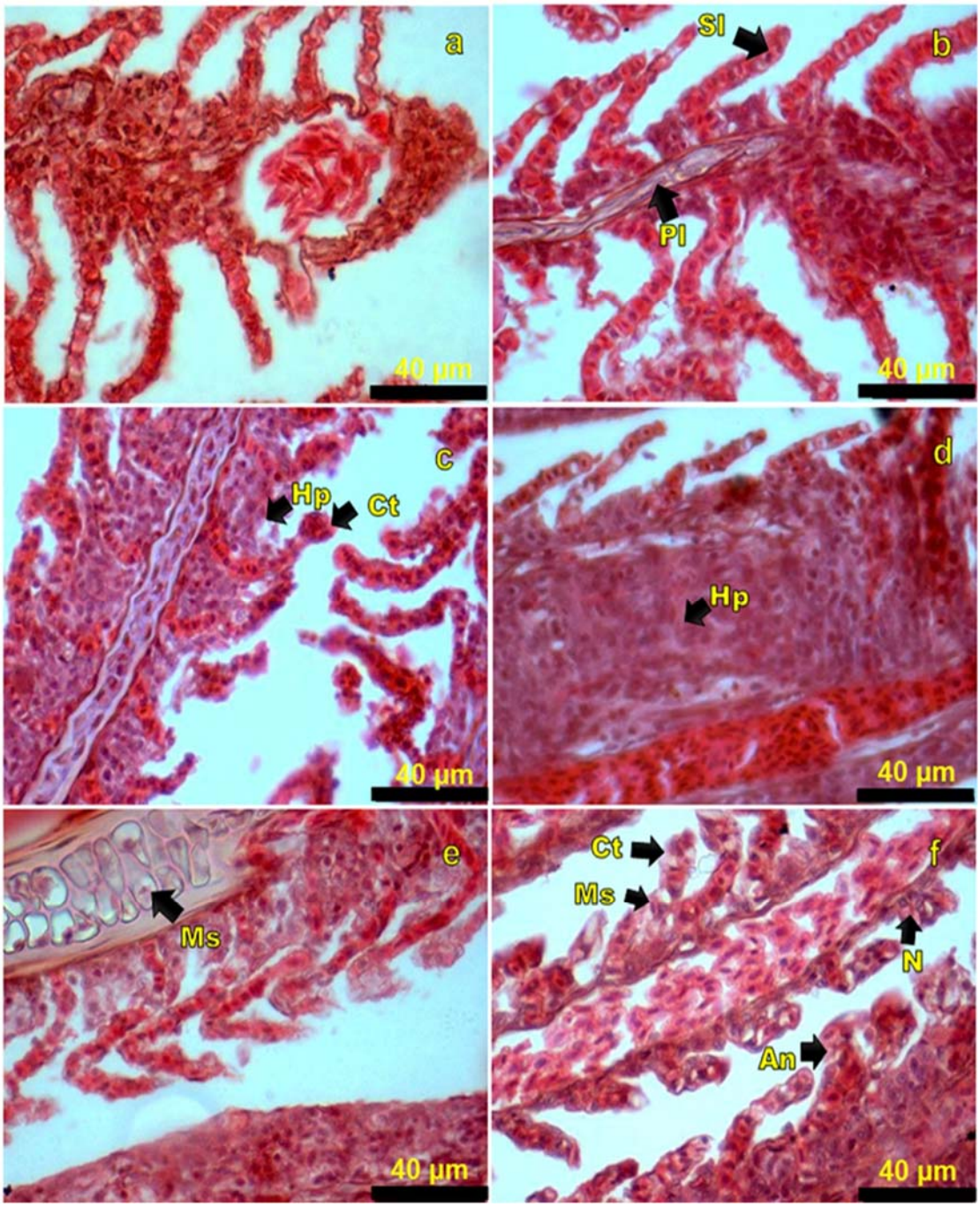

Fig 4: Histological analysis of gills in common carp following the exposure of Cu-NPs for 7 days. (a-b) Control, (c-d) lower dose and (e-f) higher dose. The gills of control fish showed normal histology normal primary lamellae, (Pl) and normal secondary lamellae (Sl) while both treatments showed injuries including include, damaged gills with necrosis (N), clubbed tips (Ct), aneurism (An), mucocytes swollen (Ms), and hyperplasia (Hp). All scale bar indicate 40 µm.

3.4 Differential regulation of liver proteome after Cu-NPs exposure

Two-dimensional electrophoresis of common carp liver between control and treated groups (100 µl/l) showed significant differences in the protein profile. Histological analysis of liver represented similar observation in both the treatments. Hence, the liver from higher dose treated group was used for the proteomic analysis. In general, several proteins were down–regulated or completely absent in the group exposed to Cu–NPs. Few up–regulated or new proteins were also observed in the exposure group (Fig. 5). The coomassie stained 2D-gels from control and treated groups were compared with Image Master 2D Platinum (GE-Healthcare) system. Out of 30 spots, 19 spots from the treated group showed downregulation in relation to the control while 11 spots showed upregulation in the treated group. These 30 spots marked for the analysis was indicated from both control and treated groups (Fig. 5). Out of 30 spots, the spots which showed significant differential expression, 4 spots (DR1, DR2, DR3, and DR4) from control gel (Fig 5a) and one (UR1) from the treated group (Fig 5b) were selected for the MALDI-TOF/TOF analysis based on low molecular weight category. This is essentially due to our aim to select few putative low molecular weight proteins to envisage the impact of Cu–NPs treatments as high molecular weight might lead to vitellogenin or its related peptides, which is predominantly analyzed in many toxicological studies. The spots identified using MALDI-TOF/TOF analysis is listed in Table 2.

Table 3: List of identified protein spots from liver tissue of common carp.

Sample	*Protein name*	*Score*	*Molecular weight in kDa*	*Calculated pI*
DR1	ferritin heavy chain	87	20450	5.26
DR2	PREDICTED: rho guanine nucleotide exchange factor 17-like	43	146858	6.03
DR3	Cytoglobin-1	37	20010	5.22
DR4	Diphosphomevalonate decarboxylase	29	45084	5.97
UR1	Selenide, water dikinase 1	37	43408	5.65

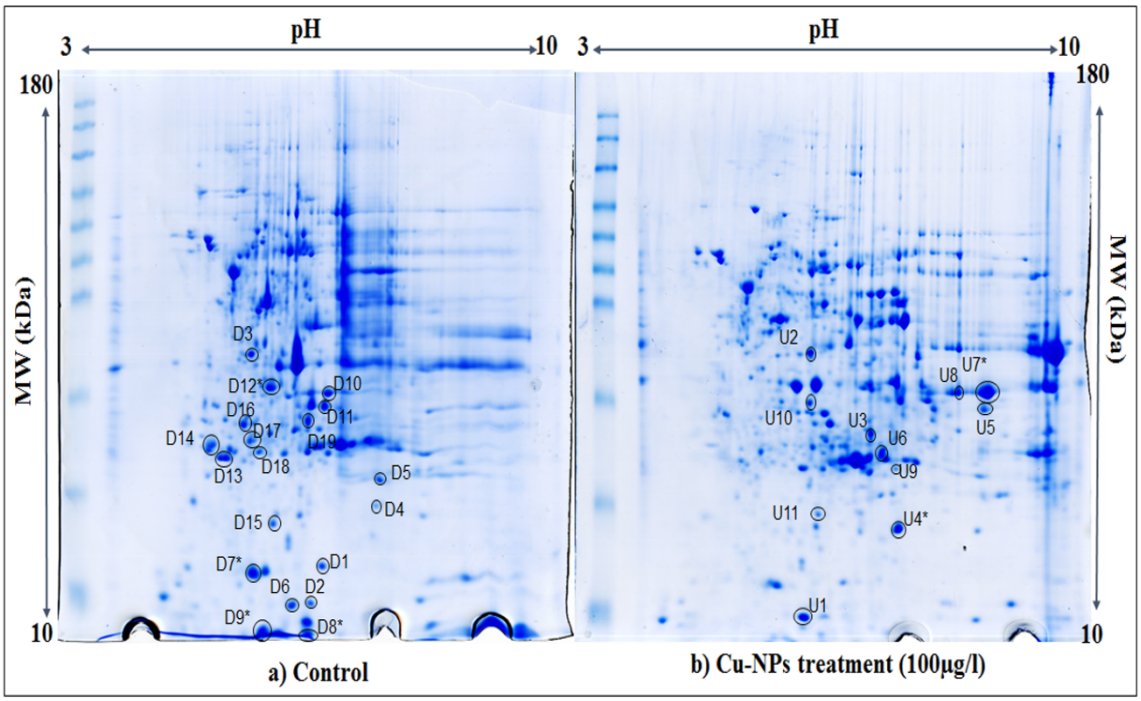

Fig 5: Two-dimensional electrophoresis gels of common carp liver following exposure of Cu-NPs for 7 days. (a) Control and (b) Cu-NPs treatment. Spots indicated by circles were upregulated (U) and downregulated (D). Spots were identified as Selenide, water dikinase 1 (UR1), ferritin heavy chain (DR1), rho guanine nucleotide exchange factor 17-like (DR2), Cytoglobin-1 (DR3) and Diphosphomevalonate decarboxylase (DR4).

3.5 Histological observation after E_2 exposure

After E_2 exposure histological analysis were performed (Fig 6A-F). At 0 dpt, gonads were undifferentiated and dominated by PGC (Fig 6A and D). At 50 dpt, stage I/II follicles were seen. There is a greater number of I/II stage follicles in E_2 exposed group when compare to control (Fig 6B and E). At 100 dpt, in control group nearly all stages follicles were seen but majority of at stage-III follicles (Fig 6C) while in E_2 exposed group mainly of stage IV follicles were seen (Fig. 6E).

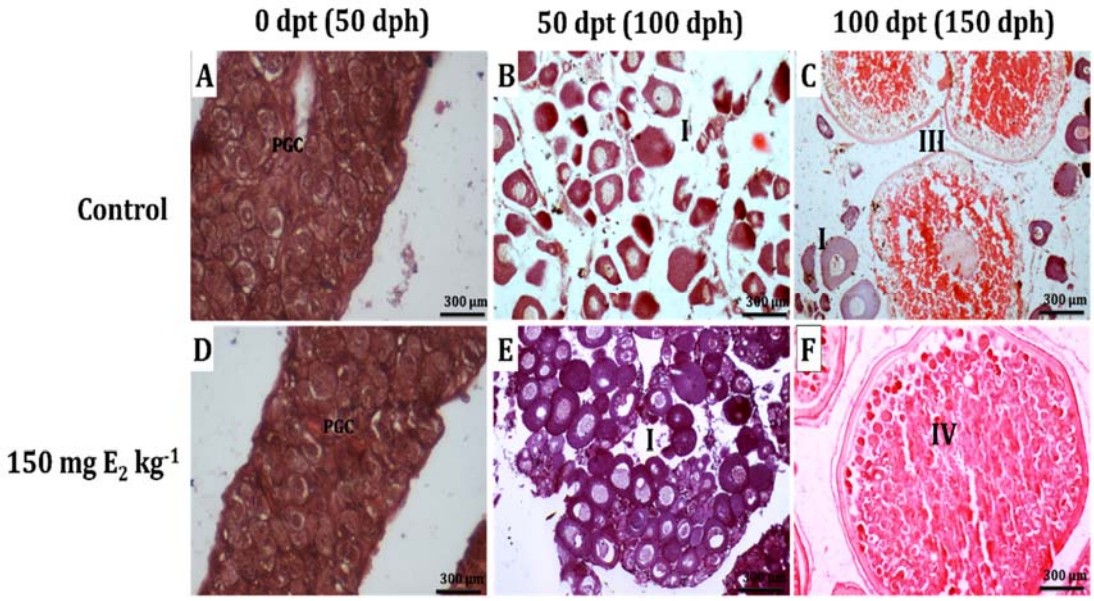

Fig 6: Histological analysis of gonads of common carp at different time point of E_2 exposure. (A-C) Control, and (D-F) E_2 exposed group. PGC: primordial germ cells, I/III/IV: stage-I/III/IV follicles, All scale bar indicate 300 µm.

3.6 Effect of E_2 on common carp gonadal gene expression profile

qPCR was performed to examine the expression pattern of *star*, *hsd3b*, *hsd17b*, *hsd20b*, *cyp19a1a*, *thoc3*, and *ptx* in E_2 fed and control groups. The expression of *star*, *hsd3b*, *hsd17b*, *hsd20b*, *cyp19a1a* was significantly higher in 150 mg E_2/kg fed group in a duration–dependent manner when compared to control. Data for 50 and 100 mg E_2/kg fed was not shown as maximum differential expression of genes were observed in 150 mg E_2/kg fed group. In addition, expression of *thoc3* and *ptx* were also found to be upregulated in 150 mg E_2/kg fed group in a duration–dependent manner (Fig. 7).

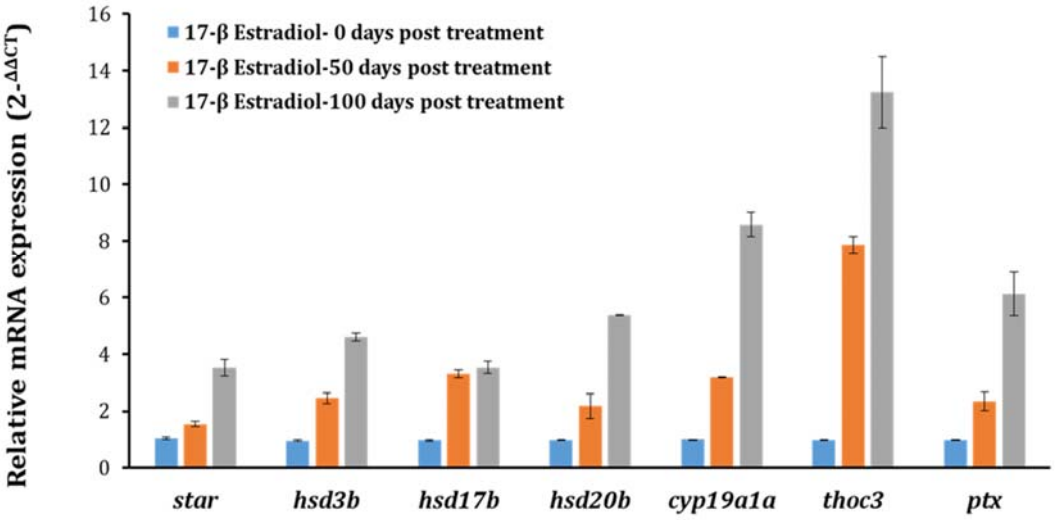

Fig. 7. Relative mRNA levels of various genes and factors such as *star*, *hsd3b*, *hsd17b*, *hsd20b*, *cyp19a1a*, which crucial role in ovarian steroidogenesis and expression pattern of *thoc3* and *ptx* after exogenous E_2. All data were expressed as mean ± SEM. Means with different letters differ significantly ($P<0.05$; ANOVA followed by Student-Newman-Keuls' post hoc test).

4 Discussion

NPs exposure to juvenile carp resulted in oxidative stress and exerts toxic effects to other tissues like kidney, liver, and gills. Proteomic data also shows alteration of several protein. Among all, protein ferritin heavy chain downregulated, having role in reproduction (Kang et al., 2015). Further, Cu-NPs could not involve in sex reversal. However, Cu-NPs causes caused testicular disarray and affected testicular development (Murugananthkumar et al., 2016) without any Cu-NPs mediated sex reversal.

In our second approach, sex steroid mediated sex reversal is well established as several forms of E_2 was used to produce monosex female populations (Smitherman and Dunham, 1985). Among all, E_2 has been one of the most effective compounds for feminization of fish (Yamazaki, 1983). Sex reversal has been accomplished by administrating the artificial

hormone by bath or feed-based (Donoldson and Hunter, 1982; Yamazaki, 1983). This method not only relies on the concentration of hormone in the diet, but also on the age of fish, duration of hormone treatment, rearing temperature, food and species used (Piferrer, 2001; Vidal-López et al., 2012). This procedure can be produced 100% sex reversal (Gorshkov et al., 2004; Wang et al., 2008). The first successful effort to artificially induce sex reversal was achieved in the medaka, through the administration of E_2 and androgens to sexually undifferentiated fish, and resulted in both functional females and males, respectively (Yamamoto, 1953, 1958). In addition, feminization study has been carried out in several fish species to demonstrate that exposure of exogenous sex steroid can efficiently influence the sex differentiation in fish towards the desired gonadal phenotype (Pandian and Sheela, 1995) irrespective of genetype.

The production of endogenous E_2 directs sexual differentiation and gonadal development. In teleost, E_2 is the principle potent hormone that promotes ovarian functions. During this, *cyp19a1a* is critical for converting testosterone in E_2 (Guiguen et al., 2010). This has been well documented in sex changing fish where the level of E_2 is high and favours ovarian development (Nakamura et al., 1989; Godwin and Thomas, 1993; Lee et al., 2001; Bhandari et al., 2003; 2006; Ohta et al., 2008; Muncaster et al., 2013). Hence, the expression of *cyp19a1a* gene (encoding aromatase, the key enzyme for E2 production) as well as ovarian steroidogenesis genes such *hsd3b*, *hsd17b*, *hsd20b* and cholesterol transporter, *star* are critical during sex reversal.

In the present study, E_2 fed fish displayed high expression of *star*, *hsd3b*, *hsd17b*, *hsd20b*, *cyp19a1a* when compared to female control. The upregulation of such genes might be due to high level of E_2 which in turn lead to the feminization of common carp. This E_2 mediated

feminization also can be validated through histological analysis. In histological analysis, the presence of a greater number of primary follicles at 50 dpt as well as matured follicle (stage-IV) at 100 dpt than control group of same dpt validated that E_2 exposure impaction it effect on ovarian steroidogenesis and growth. In addition, upregulation of *thoc3* and *ptx* were also seen which clearly suggested that *thoc3* and *ptx* did respond to high E_2 level and might have role during sex differentiation and ovarian development in carp.

Conclusions

Feminization attempt in common carp is not feasible by exposing to Cu-NPs as it caused oxidative stress, and tissue dysfunction at multiple levels. Present work demonstrated that feed-based exposure of E_2 efficiently skewed gonadal development towards the ovarian growth as evidenced by histological analysis and also upregulated the expression of *star*, *hsd3b*, *hsd17b*, *hsd20b*, and *cyp19a1a*. Correlative high expression of *thoc3* and *ptx* upon E_2 exposure validated their significant role in ovarian development and growth of common carp.

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Consolidated Summary

The current research work aimed to examine the functional role of identified factors by using DDRT approach in developing ovary of the common carp, *C. carpio*. *thoc* gene family and *ptx* were identified from 180 dph old carp developing ovary. Further, to understand its functional role in oogenesis, full length cDNA of *thoc* gene family (*thoc1*, *thoc2*, *thoc3*, *thoc5*, *thoc6*, and *thoc7*) and *ptx* was obtained by performing RACE. Further, tissue distribution showed, among *thoc* gene family, *thoc3* and *ptx* was ovary specific. Incidentally, *ptx* expression was found exclusively in ovary. Ontogeny study showed *thoc3* and *ptx* might have a role in ovarian differentiation as their expression increased from 120dph till adulthood. High expression of *thoc3* and *ptx* during prespawning and spawning phases of ovarian cycle suggested gonadotropin dependency indicating their role in follicular proliferation and ovarian growth which was confirmed by cellular layer expression as well as localization analysis. Further, *thoc3* and *ptx* significance in carp ovarian development, transient gene silencing approach was performed using carp *thoc3* and *ptx* specific custom synthesized siRNA. *In vivo* and *in vitro* transient silencing showed *thoc3* and *ptx* together promote ovarian steroidogenesis, either directly or indirectly. In addition, transient gene silencing experiments showed that *thoc3* might regulate *ptx* expression and also showed interdependency. Lastly, feminization study showed that *thoc3* and *ptx* can respond to exogeneous E_2 like that of *cyp19a1a* and other steroidogenesis-related genes, vis-à-vis ovarian development. Overall, present report suggested that both *thoc3* and *ptx* seem to be crucial for ovarian differentiation, development and maturation in common carp.

Summary of these results was represented in figure 1.

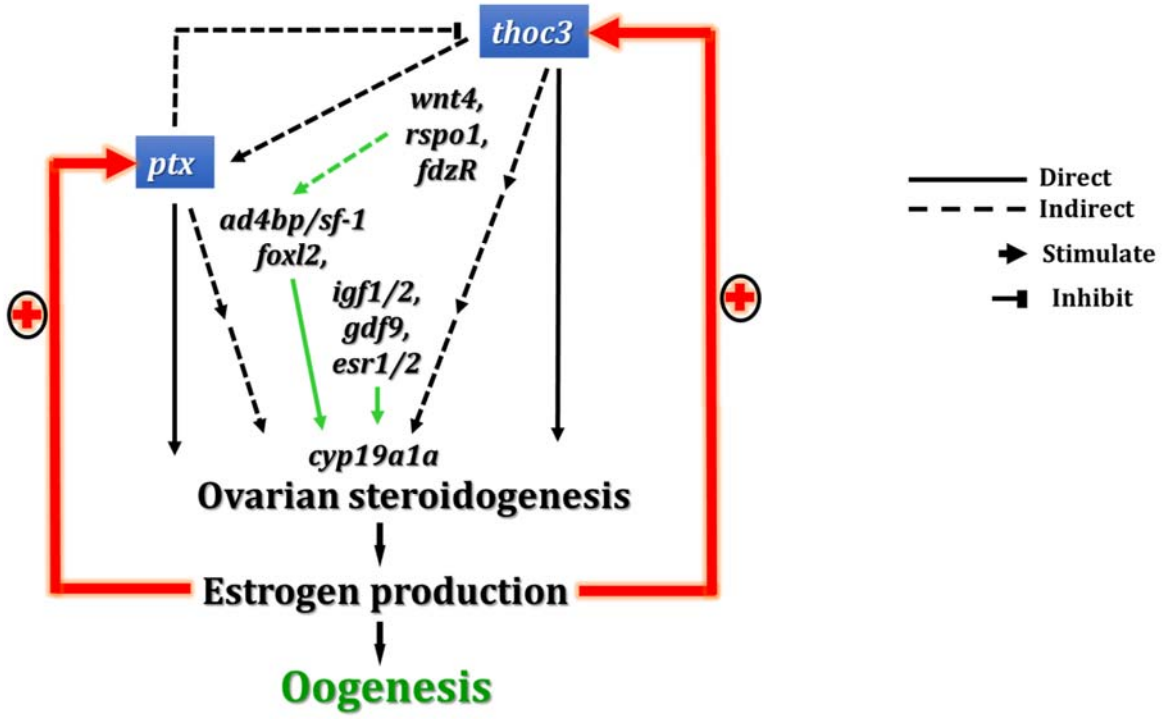

Fig. 1. Proposed mechanism of interaction of *thoc3* and *ptx* on ovarian function of common carp. In addition, their interaction with steroidogenesis during ovarian follicular growth and oocyte maturation was also shown.

Aquaculture and Fisheries 1 (2016) 15–23

Contents lists available at ScienceDirect

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journal homepage: www.keaipublishing.com/en/journals/aquaculture-and-fisheries/

Original Research Article

Effect of copper nanoparticles exposure in the physiology of the common carp (*Cyprinus carpio*): Biochemical, histological and proteomic approaches

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ARTICLE INFO

Article history:
Available online 18 October 2016

Keywords:
Catalase
Superoxide dismutase
Glutathione S-transferase
Oxidative stress

ABSTRACT

Copper nanoparticles (Cu-NPs) are serious water pollutants but their impact in teleosts performance remains poorly understood. In the present study, we have exposed juvenile carps (*Cyprinus carpio*), a freshwater teleost edible in India to two different doses (20 and 100 μg/L) of Cu-NPs for seven days. The doses selected were eco-relevant considering the contamination levels of certain water resources. The results indicated that the activity oxidative stress enzymes catalase, superoxide dismutase, and glutathione-S-transferase were significantly increased in the kidney, liver and gills of the treated groups when compared to control. Histological analysis revealed that after exposure, disruption of the secondary lamellae of gills, liver damage with pyknotic nuclei and structural disarray of the kidney occurred. Proteomic analysis of the liver showed down-regulation of several proteins including the ferritin heavy chain, rho guanine nucleotide exchange factor 17-like, cytoglobin-1 and up-regulation of diphosphomevalonate decarboxylase and selenide & water dikinase-1. Taken together, the results of suggest that short-term exposure of juvenile carp to Cu-NPs causes oxidative stress and impart serious deleterious effects in the tissues which may affect fish growth and development.

1. Introduction

Nanoparticle (NP) is a microscopic object that behaves as a whole unit with respect to its transport and properties and differs largely from bulk materials of the same composition. The properties of NPs vary with size, shape and chemical environment (Murray, Kagan, & Bawendi, 2000) and despite their widespread applications; NPs associated toxicity has gained significant importance in the last decade (De-Jong & Borm, 2008). Owing to its increased usage, there is an inevitable discharge of nanomaterials and of their byproducts from the industries in the aquatic environment, which causes adverse effects in the organisms (Mueller et al., 2012; Theron, Walker, & Cloete, 2008). Knowledge on the interaction of NPs with biotic and abiotic components is scarce and currently there is no reliable method to assess nanomaterial toxicity (Arora, Rajwade, & Paknikar, 2012; Handy, Henry, Scown, Johnston, & Tyler, 2008). However, it has been suggested that the bioavailability of metal NPs by aquatic pollution and subsequent accumulation in fish constitute a substantial risk to human health and to the environment (Shaw & Handy, 2011).

Metal oxides NPs such as copper (Cu), silver, titanium have garnered significant attention due to their negative ecological effects (Klaine et al., 2008). In fact, reports of Cu contamination were reported in several rivers and lakes of India such as Godavari river (Ghorade, Lamture, & Patil, 2014; Lokhande & Keikar, 2000). Though the contamination is minimal, separate pockets of lakes and ponds isolated from the main river stream have a high level of metallic contamination (Ghorade et al., 2014). A wide range of NPs is being used as biosensor immobilizers for greater sensitivity and specificity (Vigneshvar, Sudhakumari, Senthilkumaran, & Prakash, 2016). Due to their low preparation cost and prospective

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http://dx.doi.org/10.1016/j.aaf.2016.09.003

General and Comparative Endocrinology 279 (2019) 67–77

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Gonadal transcriptome analysis of the common carp, *Cyprinus carpio*: Identification of differentially expressed genes and SSRs☆

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ARTICLE INFO

Keywords:
Carp
Differential gene expression
Gonad
Reproduction
Sex-differentiation
Transcriptome

ABSTRACT

Common carp (*Cyprinus carpio*) is a world-wide freshwater fish of eutrophic waters. *C. carpio*, have various reproductive traits, including early sexual maturity, that may make them excellent, large, realistic, aquaculture model species. In the present work, *de novo* assembly of gonadal (testicular and ovarian) transcriptomes from juvenile common carp was performed to identify genes involved in gonadal development. A total of 81,757 and 43,257 transcripts with average lengths of 769 and 856 bp, were obtained from the immature testicular and ovarian transcriptomes, respectively. About 84,367 unigenes were constructed after removing redundancy involving representation of transcripts in both gonadal transcriptomes. Gene ontology (39,171 unigenes), clusters of orthologous group's analysis (6651 unigenes) and Kyoto encyclopedia of genes, and genomes automatic annotation server analysis (4783 unigenes) were performed to identify potential genes along with their functions. Furthermore, 18,342 (testis) and 8693 (ovary) simple sequence repeats were identified. About 298 differentially expressed genes were identified, of which 171 and 127 genes were up-regulated in testis and ovary, respectively. Quantitative real-time reverse transcription PCR was performed to validate differential expression of selected genes in testis and ovary. Nearly 809 genes related to reproduction were identified, sex-wise expression pattern of genes related to steroid synthesis, endocrine regulation, germ cell maintenance and others

Abbreviations: ad4bp/sf-1, adrenal 4 binding protein/steroidogenic factor-1; *amh*, anti-Mullerian hormone; *amhr2*, anti-Mullerian hormone receptor type 2; *amhy*, Y chromosome-linked anti-Mullerian hormone; *ar*, androgen receptor; *atm*, Ataxia telanglectasia mutated; COG, clusters of orthologous groups; Ct, cycle threshold; *ctnnbip1*, catenin beta interacting protein 1; *cux1*, cut like homeobox 1; *cux2a*, cut like homeobox 2a; *cyp11b1*, cytochrome P450, family 11, subfamily b, polypeptide 1; *cyp17*, steroidogenic cytochrome P450 17-hydroxylase/lyase; *cyp19a1*, cytochrome P450, family 19, subfamily a, polypeptide 1; *cxcl12*, C-X-C motif chemokine ligand 12; *cxcr4*, C-X-C motif chemokine receptor 4; *dax1*, dosage-sensitive sex-reversal, adrenal hypoplasia critical region, on chromosome X, gene 1; DEG, differentially expressed genes; *dmrt1*, doublesex and mab-3 related transcription factor 1; *DMY*, Y-specific DM-domain; dph, days post hatch; *egr2b*, early growth response 2b; *er*, estrogen receptor; *fam101b*, family with sequence similarity 101, member B; *fam192a*, family with sequence similarity 192, member A; *fam210b*, family with sequence similarity 210, member B; *fbox43*, F-box only protein 43; *fem1b*, fem-1 homolog B; *fgfr1a2*, fibroblast growth factor receptor 1-A-like; *foxk2*, forkhead box k2; *foxl2*, forkhead box L2; FSH, follicle stimulating hormone; *fshr*, follicle stimulating hormone receptor; *fstl3*, follistatin-like 3 (secreted glycoprotein); *fzd8*, frizzled class receptor 8; *gata4*, GATA binding protein 4; *gata6*, GATA binding protein 6; *gdf9*, growth differentiation factor 9; GnRH, gonadotropin-releasing hormone; GO, gene ontology; *gsdf*, gonadal somatic cell-derived factor; *hsd3b*, hydroxy-delta-5-steroid dehydrogenase, 3 beta; *hsd3b7*, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7 *hsd11b1* hydroxysteroid 11-beta dehydrogenase 1; *hsd11b2*, hydroxysteroid 11- beta dehydrogenase 2; *hsd17b2*, hydroxysteroid 17- beta dehydrogenase 2; *hsd20b2*, hydroxysteroid 20- beta dehydrogenase 2; *igf1ra*, insulin-like growth factor 1a receptor; *ipo4*, importin 4; KASS, KEGG, automatic annotation server; KEGG, Kyoto encyclopedia of genes and genomes; *kiss2*, kisspeptin2; *lhcgr*, luteinizing hormone/choriogonadotropin receptor; *mapk*, mitogen-activated protein kinase; *mis*, Mullerian inhibiting substance; *msl1b*, male-specific lethal 1 homolog b; NGS, next generation sequencing; *pcna*, proliferating cell nuclear antigen; *plzf*, promyelocytic leukemia zinc finger; *prlhr2a*, prolactin releasing hormone receptor 2a; PPA, protein phosphatase 2A; *pou5f1*, POU class 5 homeobox 1; qRT-PCR, quantitative real time reverse transcription PCR; RA, retinoic acid; RAR-RXR, retinoic acid receptor/retinoid x receptor heterodimers; *rspo1*, R-spondin 1; sdY, *sexually dimorphic on the Y chromosome*; *smad3b*, SMAD family member 3b; *sox9a*, SRY-box 9a; *SRY*, sex-determining region Y; SSR, simple sequence repeat; *StAR*, steriodogenic acute regulatory protein; *sycp1*, synaptonemal complex protein 1; *sycp3*, synaptonemal complex protein 3; *tac3a*, tachykinin 3a; *tcf3a*, transcription factor 3a; *TGF-β*, transforming growth factor-beta; *wnt*, wingless-type MMTV integration site family; *wt1*, Wilms tumor 1; *zar1*, zygote arrest 1; *zp2*, zona pellucida sperm-binding protein 2

☆ This work was done at University of Hyderabad.

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https://doi.org/10.1016/j.ygcen.2018.12.004
Received 27 March 2018; Received in revised form 12 December 2018; Accepted 16 December 2018
Available online 17 December 2018
0016-6480/

Involvement of thoc gene family and pentraxin in the ovarian function of Cyprinus carpio

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Submission date: 26-Jun-2019 11:38AM (UTC+0530)
Submission ID: 1147158807
File name: Yugantak_13LAPH07_merged.pdf (1.75M)
Word count: 18588
Character count: 99264

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