



## Cloning and characterization of *wnt4a* gene in a natural triploid teleost, Qi river crucian carp (*Carassius auratus*)

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### ABSTRACT

WNT4 (wingless-type MMTV integration site family, member 4) plays a key role in the ovarian differentiation and development in mammals. However, the possible roles of Wnt4 during gonadal differentiation and development need further clarification in teleosts. In this study, we cloned and characterized the full-length cDNA of Qi river crucian carp (*Carassius auratus*) *wnt4a* gene (*CA-wnt4a*). The cDNA of *CA-wnt4a* is 2337 bp, including the ORF of 1059 bp, encoding a putative protein with a transmembrane domain and a WNT family domain. Sequence and phylogenetic analyses revealed that the *CA-Wnt4a* identified is a genuine Wnt4a. Tissue distribution analysis showed that *CA-wnt4a* is expressed in all the tissues examined, including ovary. *CA-wnt4a* undergoes a stepwise increase in the embryonic stages, suggesting that *CA-wnt4a* might be involved in the early developmental stage. Ontogenic analysis demonstrated that *CA-wnt4a* expression is upregulated in the ovaries at 30–50 days after hatching (dah), the critical period of sex determination/differentiation in Qi river crucian carp. From 90 dah, the expression of *CA-wnt4a* was gradually downregulated in the developing ovaries. Immunohistochemistry demonstrated that *CA-Wnt4a* was expressed in the somatic and germ cells of the ovary by 30 dah, thereafter, positive signals of Wnt4a were detected in the somatic cells, oogonia and primary growth oocytes from 60 dah. In the sex-reversed testis induced by letrozole treatment, the expression level of *CA-wnt4a* was significantly downregulated. When *CA-wnt4a* expression was inhibited by injection of FH535 (an inhibitor of canonical Wnt/ $\beta$ -catenin signal pathway) in the ovaries, levels of *cyp19a1a*, *foxl2* mRNA were significantly downregulated, while *sox9b* and *cyp11c1* were upregulated, which suggested that together with Foxl2-leading estrogen pathway, *CA-wnt4a* signaling pathway might be involved in ovarian differentiation and repression of the male pathway gene expression in Qi river crucian carp.

### 1. Introduction

Ovarian differentiation and development is a unique and complex process which requires the integrated interaction of a network of transcription factors and growth factors during a critical period in embryogenesis. In mammals' bipotential gonads, sex determination and differentiation is controlled by an antagonistic process between the male (Sry/Sox9/Fgf9) and female (Rspo1/Wnt/ $\beta$ -catenin and Foxl2) signaling pathways (Wilhelm and Koopman, 2006; Cool and Capel, 2009; Eggers and Sinclair, 2012).

Wnt4 (wingless-type MMTV integration site family, member 4), a potent member of Wnt family, is capable of activating the canonical

Wnt/ $\beta$ -catenin signaling pathway, allowing granulosa cell differentiation and thus ovarian differentiation (Maatouk et al., 2008; Chassot et al., 2014). Abnormality of the Wnt/ $\beta$ -catenin signaling pathway resulted in masculinization of XX gonads in human, mice and goat (Vainio et al., 1999; Pailhoux et al., 2002; Parma et al., 2006; Mandel et al., 2008; Niehrs, 2012; de Lau et al., 2014). Previous reports showed that Wnt4 is highly conserved in ovarian differentiation in vertebrates, and in mammalian species, it is predominantly observed in the differentiating ovary (Pailhoux et al., 2002; Peltoketo et al., 2004; Yu et al., 2006; Jaaskelainen et al., 2010; Pask et al., 2010). Heterozygous mutations in the human *WNT4* gene caused the absence of uterine and fallopian tubes and clinical signs of excess androgen (Parma et al.,

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2006). And mice *Wnt4* deficiency also induced male-like vascularization of the embryonic XX gonad (Jeays-Ward et al., 2003), which is similar to the phenotype of mice lacking *Rspo1* (Chassot et al., 2008; Chassot et al., 2014). XY patients who have a duplication of the distal portion of chromosome 1p, which contain the *WNT4* gene, exhibit male to female sex reversal (Elejalde et al., 1984; Mohammed et al., 1989). All these studies indicate that mammalian *WNT4* is required to activate the  $\beta$ -catenin pathway to favor female ovarian differentiation and development by suppressing the expression of male sexual differentiation genes and the biosynthesis of gonadal androgen in female.

In contrast to mammals, as a consequence of the teleost-specific whole-genome duplication (3R), there are two *wnt4* genes, *wnt4a* and *wnt4b*, in most teleosts (Nicol et al., 2012). *Wnt4a*, other than *Wnt4b*, shares a high identity with tetrapod *Wnt4*, which might possess a key role in ovarian determination and development. However, in teleosts, the role of *wnt4a* in gonadal development is largely unknown. Limited expression data from rainbow trout (*Oncorhynchus mykiss*), the pro-tandrog black porgy (*Acanthopagrus schlegelii*), half-smooth tongue sole (*Cynoglossus semilaevis*), orange-spotted grouper (*Epinephelus coioides*), olive flounder (*Paralichthys olivaceus*) showed that *wnt4a* in the above mentioned teleosts varies, which showed no sexually dimorphic expression or a slightly sexual dimorphism in favor of males during gonadal differentiation (Wu and Chang, 2009; Nicol et al., 2012; Hu et al., 2014; Chen et al., 2015; Weng et al., 2016). Thus, the possible roles of *Wnt4a*, especially during gonadal differentiation in teleostean fish, need further investigation.

Qi river crucian carp (*Carassius auratus*), a local variation population of gibel carp (*Carassius gibelio*) formed by the long-term ecological adaptation, is one of the most commercially important fish since female grow faster than male and has been widely cultivated throughout the north of China (Zhou et al., 2016; Zhang et al., 2017). The fish is a unique freshwater polyploid cyprinid fish revealed to have multiple reproduction modes including unisexual gynogenesis (which is a unique characteristic different from other diploids, and will produce all-female triploid offsprings by gynogenesis when its ova are activated by heterogenous spermatozoa) and sexual reproduction (which will produce female and male individuals, with the ratio of 1:1) (Gui and Zhou, 2010). Gonad differentiation of *Carassius gibelio* occurs from 10 to 30 days after hatching (dah). And at 60 dah, the gonads develop into typical ovaries with a lot of primary oocytes (Liu et al., 2015). Therefore, the Qi river crucian carp is a good and unique model to study sex determination and differentiation in vertebrates.

The role of the *wnt4a* gene remains unknown in unisexual animals, particularly in sex determination and gonadal differentiation. Our objective was to clone and characterize the role of the *CA-wnt4a* in sex determination and differentiation. In this study, the full-length cDNA of *CA-wnt4a* was cloned and its temporal and spatial expression pattern in the gonads was studied to elucidate its possible roles in sex determination/differentiation. Moreover, the expression profiles of *CA-wnt4a* in the ovaries inhibited by injection of FH535 and also in the testis induced by letrozole (aromatase inhibitor) treatment were also examined.

## 2. Materials and methods

### 2.1. Fish

Qi river crucian carp (*Carassius auratus*) were reared in large tanks (1.2 m<sup>3</sup>) with recirculating aerated freshwater systems in the aquaculture base of Henan Normal University, Xinxiang, China. The fish were maintained at ambient temperature (24 °C) under a 14 h (h) light: 10 h dark photoperiod. In the breeding season (April to June), the selected brood fish (female Qi river crucian carp and male Common Carp at the age of 3 years old and with the weight about 400 g and 1500 g, respectively) were artificially induced into spawning by two intraperitoneal injections with a mixture of acetone-dried carp pituitary, HCG, and LRH-A (Hangzhou Animal Medicine Factory, China) (for

**Table 1**  
Sequences of primers used in the study.

primer	(5' → 3') sequence	usage
<i>wnt4a-F</i>	GCGTAATGTGGAGGTGATGG	partial sequence PCR
<i>wnt4a-R</i>	CTGCGGTTCCCAGTATGCCT	
<i>wnt4a-3' out</i>	AAGTGGGCACCACCAAGGTC	3'-RACE PCR
<i>wnt4a-3' in</i>	GCTGTGAGCTCATGTGCTGC	
<i>wnt4a-5' out</i>	CCATCACCTCCACATTACGC	5'-RACE PCR
<i>wnt4a-5' in</i>	ATCCGAGATGCTCCCCACCG	
UPM-L	CTAATACGACTCACTATAGGGC	universal primer
	AAGCAGTGGTATCAACGCAGAGT	
UPM-S	CTAATACGACTCACTATAGGGC	real-time PCR
<i>wnt4a-real-F</i>	AACGAGGCAGGAAGGAAGGC	
<i>wnt4a-real-R</i>	CTTGGTGGTGCCCACTTTGCC	housekeeping gene
<i>cyp19a1a-real-F</i>	GGTTCATCCGGTCGTGGAC	
<i>cyp19a1a-real-R</i>	TGCATCCGACCCACGTTCCAG	
<i>foxl2-real-F</i>	CACCATCACACGCACCCTCA	
<i>foxl2-real-R</i>	TGCAGCCCTGTTCCTTACC	
<i>sox9b-real-F</i>	GAGGGCGAGAAAGCGTCCATT	
<i>sox9b-real-R</i>	AGTCTCGATGAGGCCGCTCT	
<i>cyp11c1-real-F</i>	GCTGTACCTGCCTCCACGAC	
<i>cyp11c1-real-R</i>	CTCCGCGTGGCTGAAGATGT	
$\beta$ -actin-F	ACCATCTACCCCGTATTGCG	
$\beta$ -actin-R	TGGAAGGTGGACAGGGGAAGC	

females, the mixture amount of 0.4 mg + 100 IU + 2  $\mu$ g was used for the first injection, and 0 mg + 400 IU + 6  $\mu$ g for the second injection. Males were injected by one-third amount of females) (Sun et al., 2010). Under the stimulation of heterogenous sperm, the ovulated eggs of Qi river crucian carp will develop into all-female individuals through gynogenesis. The fertilized eggs were incubated at 23 °C until the embryos developed into larvae. The embryo development was observed regularly during incubation. All animal experiments conformed to the Guide for Care and Use of Laboratory Animals and euthanasia is performed by immersion fish in MS-222 solution, and all efforts were made to minimize suffering.

### 2.2. Molecular cloning and sequence analysis

According to the highly conserved regions of teleost *wnt4a* homologs, a pair of primers (*wnt4a-F* and *wnt4a-R*, in Table 1) was designed to amplify the target fragment of the *wnt4a* cDNA. Full-length *wnt4a* cDNA was obtained by 5'- and 3'-RACE method using the SMARTer™-RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's protocol. The 5'- and 3'-RACE primers (Table 1) were designed based on the obtained *wnt4a* cDNA fragment sequences. The PCR program was performed for 37 cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 2 min. All PCR products were ligated into the pGEM-T easy vector (Promega, USA) and sequenced at Life Technologies Corporation (Shanghai, China). The cDNA sequence has been submitted to GenBank with accession no. MK118724. Multiple alignments of *Wnt4* proteins were performed with BioEdit software. The neighbor-joining method was used to construct the phylogenetic tree by Mega 5.0 (Tamura et al., 2011) by using zebrafish *Wnt5* as an out-group.

### 2.3. Real-time PCR and statistical analysis

The primer sets used for real-time PCR were designed using Primer Express software (Applied Biosystems, USA). Liner standard curves were generated with serial 10-fold dilutions with plasmids DNA containing the ORF of the target gene and the internal control gene  $\beta$ -actin to detect the primer efficiency, the primer sets with the efficiency between 90 and 110% were used for real-time PCR analysis. All real-time PCRs were carried out in a LightCycler 96 Real-time PCR machine (Roche, Switzerland) in a 20  $\mu$ l reactions with a mixture of 10  $\mu$ l 2 × SYBR Premix ExTaq (TaKaRa, Japan), 2.0  $\mu$ l of diluted cDNA or PCR-grade water as negative control, 6  $\mu$ l of PCR-grade water, and

1.0  $\mu$ l of each 10  $\mu$ M primer. The PCR reactions were initiated by denaturation at 95 °C for 5 min; followed by 40 amplification cycles at 95 °C for 15 s and 60 °C for 30 s. Dissociation protocols were used to measure melting curves. The relative expression level (RNA abundance) was calculated by dividing the copy number of the target gene by that of the internal control gene. Data are expressed as the mean  $\pm$  SD. Significance ( $P < 0.05$ ) in the results was determined using the Student's *t*-test or one-way ANOVA followed by Bonferroni post hoc test as appropriate for single or multiple comparisons respectively.

#### 2.4. Tissue distribution of *CA-wnt4a*

For the tissue distribution analysis, three independent samples for each tissue were prepared to evaluate the expression of *CA-wnt4a* gene. Briefly, total RNA was extracted from brain, gill, head kidney, heart, liver, intestine, spleen, kidney, muscle and ovary of adult Qi river crucian carp, according to the manufacturer's instructions (TaKaRa, Japan). Total RNA (500 ng) from various tissues was respectively reverse transcribed into first-strand cDNA using PrimeScript RT Master Mix Perfect Real Time Kit (TaKaRa, Japan) according to the manufacturer's instructions. Finally, real-time PCR was carried out to check the expression level of *CA-wnt4a*, according to the aforementioned method.

#### 2.5. *CA-Wnt4a* expression during embryogenesis and ontogeny

Three independent samples from 10 embryonic fish for each stage (unfertilized eggs, 2-cell stage, blastula, gastrula, neurula, tail-bud, heart-beating, and hatching stages) were used for embryogenesis expression analysis. Gonad differentiation of *Carassius gibelio* were previously observed to occur within 20–30 dah, and oögonia were differentiated and proliferated in the enlarged gonads at 45 dah, then at 60 dah, the gonads had developed into typical ovaries with primary growth stage oocytes (Xia et al., 2007; Peng et al., 2009; Liu et al., 2015). For ontogenic expression analysis to be carried out in the present study, three independent gonadal samples from 40 to 80 fish were pooled for each sample collected from 15 to 50 dah. One ovary was used for each sample collected from 60, 80, 90, 120, 150 and 720 dah. RNA extraction, cDNA synthesis, real-time PCR and statistical analysis were carried out as described above.

#### 2.6. Cellular localization

To detect the protein localization of *CA-Wnt4a*, the antibody against human WNT4 was purchased from CUSABIO (China, P56705). The antibody was generated from a 329-residue polypeptide (23–351) of human, which shares 83% amino acid homologues with *CA-Wnt4a*. Before IHC analysis, the specificity of the antibody in Qi river crucian carp was characterized by western blotting as described previously (Yu et al., 2014). For IHC analysis, the whole bodies after removal of the yolk and gut of Qi river crucian carp fry at 25, 30, 40, 60 dah, gonads of 70, 150, 210 and 720 dah were dissected, fixed in Bouin's solution for 12 h at room temperature. Then, the tissues were dehydrated, embedded in paraffin and sectioned at 7  $\mu$ m. Subsequently, the sections were deparaffinized, hydrated, and treated with a blocking solution, then incubated with the primary polyclonal antibody (diluted 1:500) overnight at 4 °C, and rinsed with 1  $\times$  PBS three times for 5 min. The sections were then incubated with a secondary antibody conjugated with horseradish peroxidase (diluted 1:2000; Bio-Rad) for 30 min, washed with PBS, and visualized with 3, 3'-diaminobenzidine (Sigma-Aldrich, Germany). Finally, the sections were counterstained with hematoxylin. For the negative control, the primary antibody was replaced with normal rabbit serum. In this study, all the IHC images were acquired with a Zeiss Axio Scope A1 microscope equipped with an AxioCam MRc5 digital camera.

#### 2.7. Gene expression in the ovaries after treatment with FH535

Since FH535 (Sigma-Aldrich, Germany) acts as a potent inhibitor of the Wnt/ $\beta$ -catenin signal pathway (Handeli and Simon, 2008; Liu et al., 2016), it was selected to study the roles of Wnt4/ $\beta$ -catenin signal pathway in Qi river crucian ovary. For the experiments, FH535 was diluted in 0.5% DMSO and injected into the gonads of 100 dah fish at a dosage of 25 mg/kg/day as reported previously (Ren et al., 2016; Zhu et al., 2017). The injections were given twice, once every 24 h. The control group of 15 individuals was treated with an equal volume of 0.5% DMSO. Finally, the ovaries of both control and treated fish were collected to examine the expression of *wnt4a*, *cyp19a1a*, *foxl2*, *sox9b* and *cyp11c1* by real-time PCR at 48 h after treatment.

#### 2.8. Effect of letrozole treatment on the expression of *CA-Wnt4a*

It is well known that inhibition of *cyp19a1a* gene by letrozole treatment during the critical window of sex differentiation results in sex reversal in teleosts (Kitano et al., 2000; Kobayashi et al., 2003; Tzchori et al., 2004; Sun et al., 2007). In this study, the expression of *CA-wnt4a* was evaluated in letrozole induced sex-reversed testis. In brief, Qi river crucian carp larvae were reared in aerated fresh water containing letrozole (Sigma-Aldrich, Germany) (10 ng/L) dissolved in ethanol from 5 to 60 dah with 50% water exchange daily. Fish reared in ethanol treatment and normal water were used as vehicle control and negative control, respectively. Then the fish were normally reared until sampling at 210 dah. Three independent samples, each contained at least 3 fish gonads, were prepared for the three groups i.e. vehicle control and negative control, letrozole treated group. Subsequently, RNA extraction, cDNA synthesis and real-time PCR were carried out to investigate the expression profiles of *CA-wnt4a* gene. Additionally, gonadal samples for IHC analysis were also prepared to further confirm the expression changes of *CA-Wnt4a* after letrozole treatment.

Primer sequences used in the present study were listed in Table 1.

### 3. Results

#### 3.1. Cloning and characterization of *CA-wnt4a* cDNA

Using degenerated primers and RACE-PCR strategy, we cloned the full-length cDNA of *CA-wnt4a*. *CA-wnt4a* cDNA (accession number: MK118724) was 2337 bp in length containing an open reading frame (ORF) of 1059 bp, corresponding to 352 amino acid residues and the 5' and 3' UTRs of 348 and 930 bp, respectively (Fig. S1). The predicted molecular mass and pI value of the deduced *CA-Wnt4a* protein were 39 kDa and 8.61, respectively.

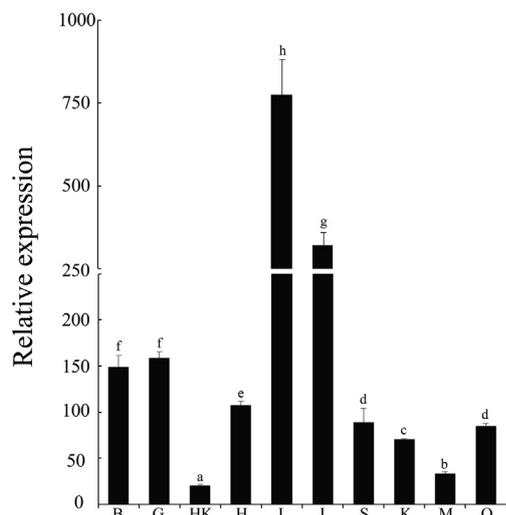
#### 3.2. Sequence and phylogenetic analyses

Sequence analysis showed that *CA-Wnt4a* contained a transmembrane region and a WNT1 domain, an important feature of Wnt gene family (Fig. S2). Alignment of the *CA-Wnt4a* with those from other organisms revealed a high identity, especially in the WNT1 domain. The overall similarity between *CA-Wnt4a* and its counterparts in other fish species at the amino acid level, was about 89–99%, and 82–87% with those from tetrapods (Fig. S2).

A phylogenetic tree was constructed to understand the phylogenetic relationships of the Wnt4 proteins among the vertebrates. It showed that *CA-Wnt4a* was clustered into one clade with Wnt4 from amphibian, reptile, and mammalian homologues, while fish Wnt4b formed a distinct fish specific clade (Fig. S3).

#### 3.3. Tissue distribution

A real-time PCR analysis was used to investigate the expression profiles of *CA-wnt4a* in the adult tissues. The results showed that *CA-*



**Fig. 1.** Tissue distribution of *CA-wnt4a* by real-time PCR. B, brain; G, gill; HK, head kidney; H, heart; L, liver; I, intestine; S, spleen; K, kidney; M, muscle; O, ovary. Data are expressed as the mean  $\pm$  SD of three independent samples. Different lowercase indicates significant difference.

*wnt4a* was ubiquitously expressed in almost all the tissues examined, with higher levels in the liver, and intestine; moderate levels in the brain, gill, heart, spleen, kidney and ovary; relatively lower levels in the muscle and head kidney (Fig. 1).

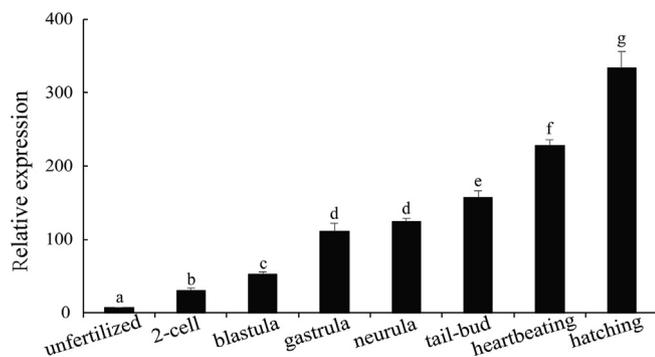
### 3.4. Temporal expression of *CA-wnt4a* during embryogenesis

Furthermore, we used real-time PCR analysis to detect the temporal expression of *CA-wnt4a* during embryogenesis. As shown in Fig. 2, *CA-wnt4a* undergoes a stepwise increase in the embryos from 2-cell stage, and reaches a high level at hatching stage.

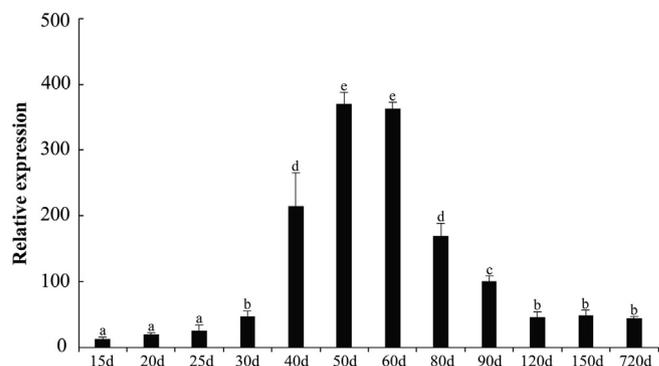
### 3.5. Ontogenic expression of *CA-wnt4a* in female gonads

Real-time PCR demonstrated that *CA-wnt4a* increased from 30 dah, and between 40 and 50 dah (the critical period of ovarian differentiation in Qi river crucian carp), it increased rapidly, and continued to increase gradually up to 60 dah, reached the highest level, after which there was a gradually decreases, and only low level was detected from 120 dah, and then *CA-wnt4a* expression remained low throughout the late ovarian developmental stages (Fig. 3).

By IHC, positive signals of *CA-Wnt4a* were detected in somatic cells of the ovary at 30 dah (Fig. 4C). At 40 dah, positive signals were detected in the meiotic germ cells and somatic cells (Fig. 4E). At later stages (60 and 70 dah), a positive signal of *Wnt4a* was detected in the



**Fig. 2.** Expression of *CA-wnt4a* during embryogenesis. Data are expressed as the mean  $\pm$  SD of three different embryonic pools at each developmental stage. Different lowercase indicates significant difference ( $P < 0.05$ ).



**Fig. 3.** Expression of *CA-wnt4a* in the Qi river crucian carp ovaries at different developmental stages by real-time PCR. Data are expressed as the mean  $\pm$  SD of three different gonadal pools at each developmental stage. Different lowercase indicates significant difference ( $P < 0.05$ ). d, days after hatching.

oogonia, stage I oocytes and somatic cells (Fig. 4G, I); and from 150 to 720 dah, the signal of *Wnt4a* was only detected in oogonia and some somatic cells (Fig. 4K, M, O). While no signal was detected in the 25 dah, and negative controls gonads (Fig. 4A, B, D, F, H, J, L, N, P). Western blotting result showed that the WNT4 antibody could recognize a specific band about 40 kDa in Qi river crucian carp (Fig. S4).

### 3.6. FH535 treatment downregulated *CA-wnt4a*, *cyp19a1a*, *foxl2* and upregulated *sox9b*, and *cyp11c1* expression

After 48 h of FH535 treatment, all fish were healthy and no adverse effects were observed. The transcription of the *CA-wnt4a* was significantly suppressed at 24 and 48 h by FH535 treatment (Fig. 5A), and the treatment time was determined as 48 h to compare the suppression effects on other sex-biased genes. At 48 h, the expression of *cyp19a1a* and *foxl2* was also downregulated in the ovaries (Fig. 5B), while the expression of male marker genes *sox9b*, and *cyp11c1* was activated (Fig. 5B).

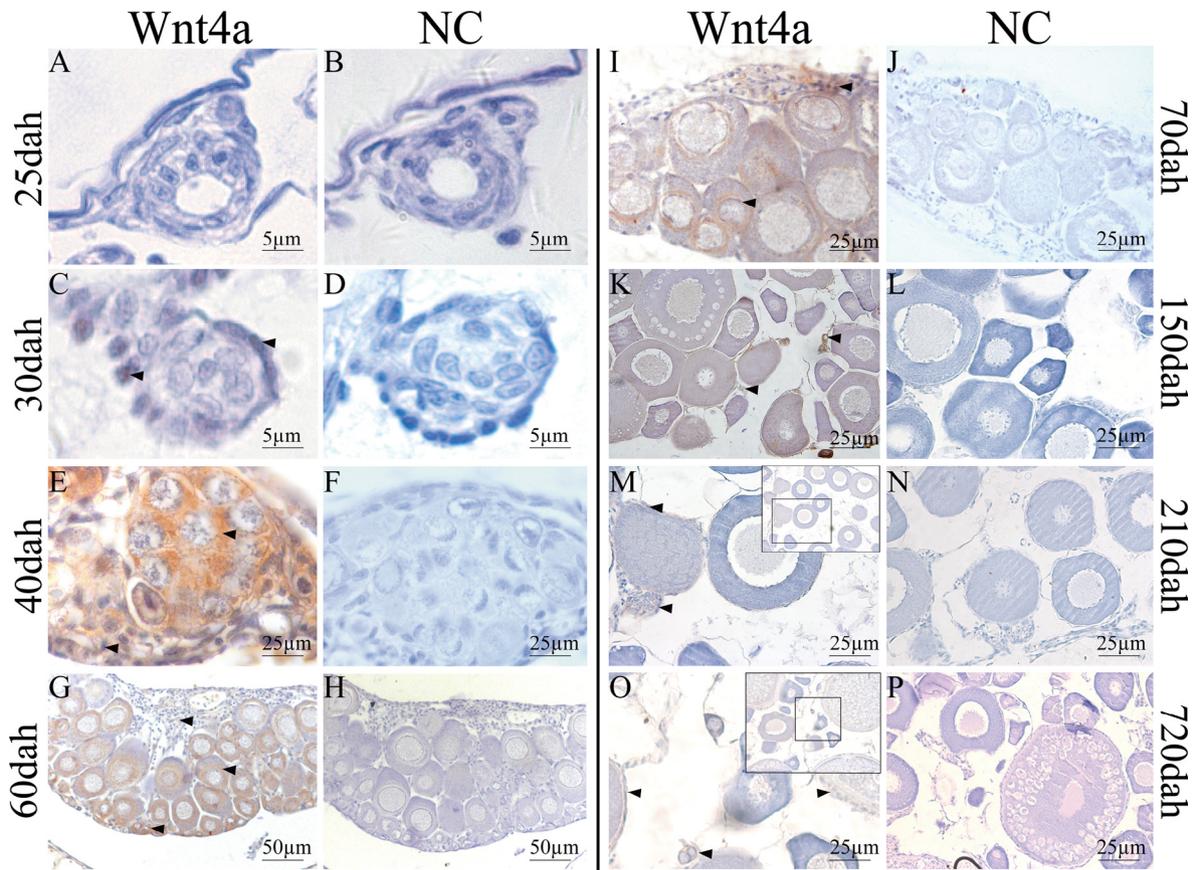
### 3.7. Effects of letrozole treatment

Histologically, treated Qi river crucian carp with letrozole for about 60 d resulted in testis formation (200 larvae were used in both control and treated groups, and the sex reversal rate is about 98%) (Fig. 7). Real-time PCR analysis revealed that *CA-wnt4a* was significantly downregulated in the sex-reversed fish gonad (Fig. 6), while no significant difference was observed between the vehicle and the negative control group (data not shown). By IHC, *CA-Wnt4a* was detected in the spermatogonia and spermatocytes of the testis (Fig. 7). No signal was detected in the negative control.

## 4. Discussions

As a component activator of canonical Wnt/ $\beta$ -catenin signaling pathway, WNT4 plays a key role in mammalian sex determination, reproduction, and sex inversion (Kim et al., 2006; Inohaya et al., 2010; Janssen and Posnien, 2014). To detect its role in teleost ovarian differentiation, we cloned the *CA-wnt4a* cDNA, investigated its tissue distribution, ontogenic expression profile and letrozole induced gene expression. At the same time, gene expression patterns were also studied when the canonical Wnt signal pathway was blocked by FH535 injection. Our data demonstrated that together with *Foxl2*-leading estrogen pathway, *CA-wnt4a* signaling pathway might be involved in ovarian differentiation and repression of the male pathway gene expression in Qi river crucian carp.

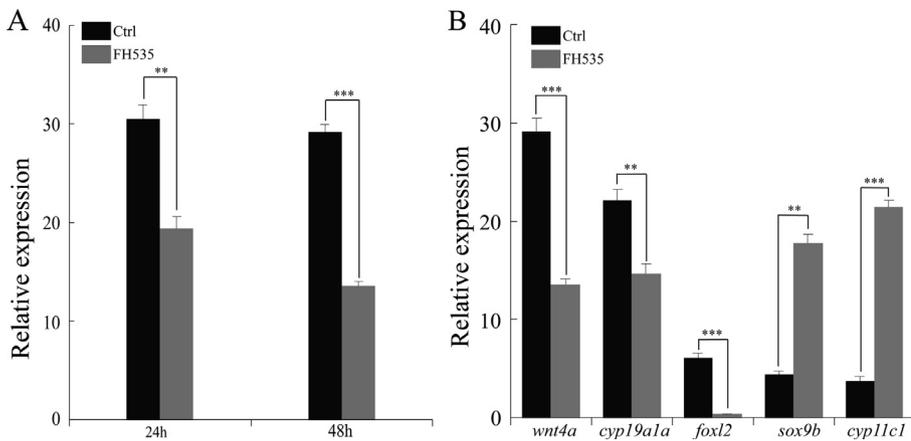
Phylogenetic analysis showed that there were two *Wnt4* in teleosts (*Wnt4a* and *Wnt4b*), with *Wnt4a* clustered together with *Wnt4* from



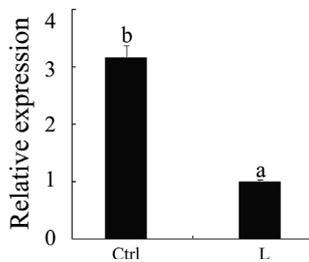
**Fig. 4.** Cellular localization of CA-Wnt4a in Qi river crucian carp ovaries at different ontogenic stages by immunohistochemistry. At 30 dah, positive signals were detected in the somatic cells. From 40 dah on, positive signals were detected in the somatic cells, oogonia, and primary growth oocytes (E, G, I, K, M, O). Arrowheads indicate the positive signal. dah, days after hatching; NC, negative control.

tetrapods, indicating that CA-Wnt4a is the homologue of mammalian counterparts' Wnt4. Moreover, sequence analysis showed that CA-Wnt4a shared the characteristic features with mammalian WNT4 and Wnt4a from other teleosts, ie the putative conservative domain of WNT1 and the glycosylation sites. All the results indicated that the CA-Wnt4a we cloned is the genuine Wnt4a. Previous reports demonstrated that the two *wnt4* subtypes showed distinct temporal and spatial expression patterns in teleosts (Ungar et al., 1995; Liu et al., 2000; Yokoi et al., 2003; Inohaya et al., 2010; Nicol et al., 2012; Chen et al., 2015), and previous study in medaka demonstrated their functional divergence (Inohaya et al., 2010), suggesting different physiological roles of the two *wnt4* genes, with Wnt4a possessing a conserved role in ovarian differentiation in fish.

CA-*wnt4a* showed a stepwise increase during the embryonic developmental stage, which is in agreement with what has been previously described in other teleosts, including rainbow trout, zebrafish, and medaka (Ungar et al., 1995; Yokoi et al., 2003; Matsui et al., 2005; Inohaya et al., 2010; Nicol et al., 2012), indicating its conserved roles in embryonic development. In adult individuals, CA-*wnt4a* was ubiquitously expressed in almost all the tissues examined, with higher levels in the liver and intestine; moderate levels in the brain, gill, heart, spleen and ovary. The expression of CA-*wnt4a* in the ovary indicated its possible role in the maintenance of ovarian development, which is consistent with observations in zebrafish, rainbow trout, and half-smooth tongue sole (Matsui et al., 2005; Nicol et al., 2012; Hu et al., 2014). Moreover, the ontogenetic expression data showed that during the



**Fig. 5.** FH535 treatment on gene expression in Qi river crucian carp ovary. A, Expression of CA-*wnt4a* at 24 and 48 h in the ovary after injection with FH535. B, Expression of CA-*wnt4a*, *cyp19a1a*, *foxl2*, *sox9b*, and *cyp11c1* 48 h after injection with FH535. Data are expressed as the mean  $\pm$  SD of six different gonadal samples. Asterisks indicate significant difference ( $P < 0.05$ ) as determined by Student's *t*-test. FH535, antagonist of canonical Wnt/ $\beta$ -catenin signal pathway.



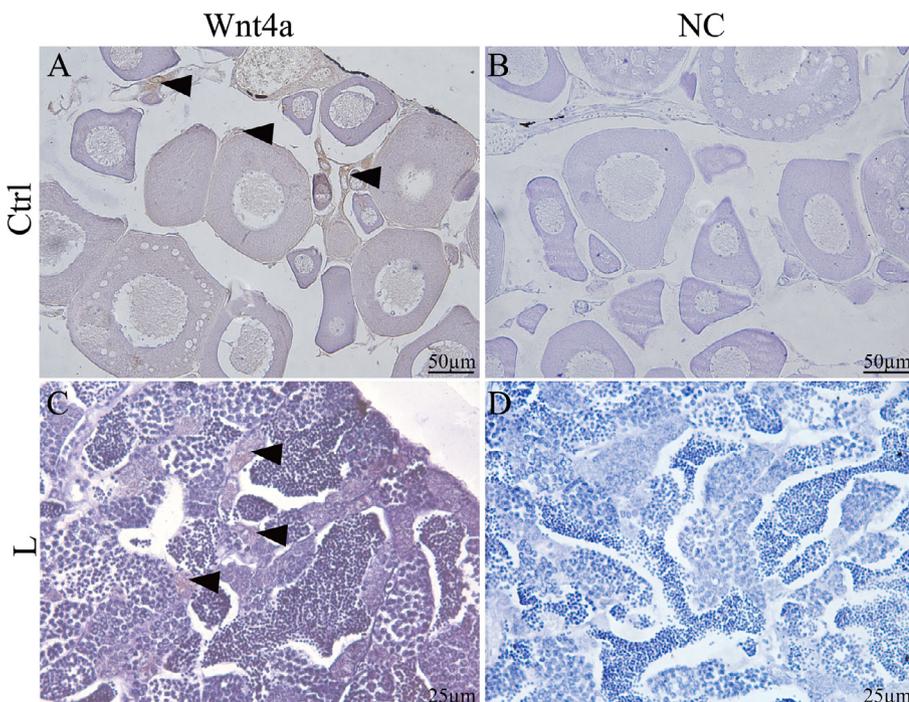
**Fig. 6.** Letrozole treatment on *CA-wnt4a* expression in Qi river crucian carp gonad by real-time PCR. In letrozole treated sex-reversed testis, the expression of *CA-wnt4a* decreased significantly ( $P < 0.05$ ). Data are expressed as the mean  $\pm$  SD of four different gonadal samples. Different lowercase indicates significant difference ( $P < 0.05$ ) as determined by Student's *t*-test. Ctrl, control; L, letrozole.

critical period of ovarian determination and differentiation, a significant increase in the expression of *CA-wnt4a* just before meiotic initiation (30 dah) in the ovary, and restriction to the germ cells of the ovary from 40 dah, suggesting that the Wnt4-activated signaling pathway might be involved in the entry into meiosis in Qi river crucian carp. And after 60 dah, the expression level decreased and, remained at a relatively lower level. The results are in accordance with studies in orange-spotted grouper, half-smooth tongue sole, black porgy and rainbow trout (Wu and Chang, 2009; Nicol et al., 2012; Chen et al., 2015), indicating the involvement of *wnt4a* in early ovarian formation and meiotic initiation. The moderate level of *wnt4a* in adult ovary might be due to the decrease after the ovarian differentiation, but the possibility can't be excluded that the activity of Wnt4 in adult ovary might be higher.

The cellular localization of Wnt4 in the somatic cell of differentiating gonad seems to be well conserved from amphibians to mammals (Vainio et al., 1999; Hsieh et al., 2002; Oreal et al., 2002; Yao et al., 2004; Yu et al., 2006; Shoemaker et al., 2007; Jaaskelainen et al., 2010). Following sex differentiation, Wnt4 expression was detected in the granulosa cells of small follicles in mice, human, and chickens (Hsieh et al., 2002; Oreal et al., 2002; Jaaskelainen et al., 2010). In the present study, *CA-Wnt4a* was detected in the somatic cell of the pre-differentiation ovary and then mainly in the oogonia, meiotic oocyte of

the differentiation ovary, which is in accordance with the results of black porgy and half-smooth tongue sole (Wu and Chang, 2009; Hu et al., 2014). Considering the cellular localization and critical timing of *CA-Wnt4a* expression, our data further strengthen the essential roles of Wnt4-activated signaling pathway in female sex determination and ovarian differentiation in fish. However, expression of Wnt4a in letrozole-induced sex-reversed testis indicated that Wnt4a might be also required for testicular differentiation in teleosts as reported previously (Hu et al., 2014).

The role of Wnt4 during ovarian differentiation is often described as an anti-testis factor that antagonizes the action of testis-specific genes (Kim et al., 2006). Activation of the  $\beta$ -catenin signaling pathway in mammalian XX gonad by *Rspo1* and/or Wnt4 is required for granulosa cell differentiation and thus ovarian differentiation (Maatouk et al., 2008; Chassot et al., 2014). Loss of either WNT4 or RSPO1 in XX mice resulted in partial sex reversal, with the appearance of ectopic androgen-producing cells, increased male-specific genes expression and androgen levels, and oocyte depletion (Jeays-Ward et al., 2003; Chassot et al., 2008; Chassot et al., 2012; Chassot et al., 2014). The findings indicate the critical functions of WNT/ $\beta$ -catenin signal pathway during ovarian differentiation in mammals. However, there has been little information regarding the functional studies of the Wnt signaling pathway in the gonadal differentiation of teleosts. Our previous reports in tilapia demonstrated that knockdown of  $\beta$ -catenin or *Rspo1* in the XX gonad caused the masculinization of the ovary; while overexpression of *Rspo1* in XY medaka caused the complete sex reversal (Wu et al., 2016a,b; Zhou et al., 2016). Inhibition of  $\beta$ -catenin by injection of quercetin in the ovary of half-smooth tongue sole, the expression of *foxl2* was significantly downregulated, and *dmrt1* was upregulated. In this study, blockage of Wnt/ $\beta$ -catenin signaling pathway by FH535 treatment resulted in the significant downregulation of female-biased genes of *wnt4a*, *cyp19a1a*, *foxl2*, and upregulation of male-biased genes of *sox9b* and *cyp11c1*, indicating that Wnt4-activated signaling pathway might be essential for proper ovarian differentiation, estrogen production and antagonizing the male pathway gene expression in Qi river crucian carp. Considering these data together, we infer that despite the diversity of sex determinants in different phyla, the Wnt/ $\beta$ -catenin pathway is required to promote ovarian development by antagonizing the expression of male pathway genes in the female gonad.



**Fig. 7.** Letrozole treatment on *CA-Wnt4a* expression in Qi river crucian carp gonad by immunohistochemistry. In control ovary, positive signal of Wnt4a was detected in the oogonia and somatic cells, and in the sex-reversed testis, positive signal of Wnt4a was detected in the spermatogonia and spermatocyte. Arrowheads indicate positive signal. Ctrl, control; L, letrozole; NC, negative control.

Estrogen is considered a natural inducer of ovarian differentiation during early female sex determination/differentiation in fish (Nagahama, 2005). Inhibition of *cyp19a1a* gene by letrozole treatment during the critical window of sex differentiation resulted in sex reversal in teleosts (Kobayashi et al., 2003). Treatment Qi river crucian carp with letrozole for about 60 days during the critical period of sex determination and differentiation resulted in sex reversal. The expression of *CA-wnt4a* was significantly downregulated in neomales. Therefore, blockage of estrogen production by letrozole treatment resulted in the inhibition of Wnt4-activated signaling pathway, which might promote the sex reversal in gynogenesis Qi river crucian. These data further proved estrogen pathway synergized with Wnt4-activated signaling pathway to ensure ovarian differentiation in fish.

In summary, our data suggest that the fish Wnt4a signaling pathway is involved in the entry into meiosis during early ovarian differentiation, and also favor the ovarian fate by antagonizing the expression of testis developmental pathways genes. Despite the diverse sex determination mechanisms in different phyla, WNT/ $\beta$ -catenin signaling appears to be an ancient, conserved pathway of ovarian differentiation among vertebrates.

#### Declaration of interest

The authors declare that there is no financial or other potential conflict of interests.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ygcen.2019.03.016>.

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