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Identification of *suh* gene and evidence for involvement of notch signaling pathway on gonadal differentiation of Yellow River carp (*Cyprinus carpio*)

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Abstract The *suh* gene is crucial in Notch pathway and regulates mammalian gonad development. In this study, the sequences of *suh1* and *suh2* genes in Yellow River carp (*Cyprinus carpio*) were verified. The partial 5′-flanking regions of *suh1* and *suh2* were analyzed and several potential transcription factor-binding sites were identified. Phylogenetic, gene structure, and chromosome synteny analyses revealed that carp *suh1* and *suh2* were orthologs and homologous to vertebrate *suh*. Investigation of the expression profiles of *suh1* and *suh2* with qPCR showed that these genes were abundant in the brain and gonad of carp, with *suh1* exhibiting sexual dimorphism expression pattern in gonad. To study the relationship between gonad differentiation and Notch signaling, primordial gonads were exposed to DAPT, an inhibitor of Notch signaling, in vitro and in vivo. The results revealed a significant downregulation of *suh1* and other Notch genes in vitro. In addition, expression of male-biased genes, such as *amh*, *dmrt1*, etc., was downregulated, whereas that of female-biased genes, such as *foxl2*, *gdf9*, etc., was upregulated. When the primordial gonads were subjected to long-term DAPT exposure, an increased proportion of ovary and delay in testis development were observed. These results suggest that *suh* gene may have a conservative function between teleosts and mammals. Furthermore, Notch signaling was found to be involved in gonad differentiation in Yellow River carp, and DAPT was noted to inhibit and enhance the expression of male- and female-biased genes, respectively, and induce the increase in number of females.

Keywords suh1 · suh2 · Notch signaling pathway · DAPT · Gonadal development · Yellow River carp (*Cyprinus carpio*)

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Introduction

Notch gene was first identified by Morgan in 1917 and was named after the wing edge notch observed in fruit fly (*Drosophila melanogaster*) following deletion of this gene. In 1983, Artavanis-Tsakonas first cloned the Notch gene, which encodes a large class of transmembrane receptors (Artavanis-Tsakonas et al., 1983). The Notch signaling pathway is a highly conserved cell signaling system present in most of the vertebrates and invertebrates. In mammals, four Notch family receptors, encoded by *notch1*, *notch2*, *notch3*, and *notch4* genes, have been described. The Notch ligands are encoded by *Jagged1*, *Jagged2*, *Deltalike1*, *Deltalike3*, and *Deltalike4* genes. When Notch ligands bind to Notch receptors, the receptors become susceptible to proteolytic cleavage mediated by γ-secretase complex, which is a large protease complex composed of a

catalytic subunit (*psen-1* or *psen-2*) and accessory subunits (*pen2*, *aph1*, and *nicastrin*), for releasing the intracellular domain of Notch (NICD) (Struhl and Greenwald 2001; Greenwald 2012; Jarriault et al. 1995). The NICD then translocates into the nucleus, where it interacts with the *suppressor of hairless* (*suh*) gene, which is a transcription factor (TF) known as recombination signal binding protein for immunoglobulin kappa J region (RBPJ) (Jarriault et al. 1995). When the NICD binds to *suh*, the gene switches from being a repressor to an activator of transcription and regulates hairy and enhancer of split (*Hes*) and *Hes*-related YRPW motif TF (*Hey*), which are Notch target genes (Struhl and Greenwald 2001; Jarriault et al. 1995). These target genes function as transcriptional factors to regulate the expression of other genes in different cells. In previous studies, Notch signaling was observed to be involved in gonad development and assembly of primordial follicle (Vanorny et al. 2014; Wang et al. 2015), and *suh* knockout mice exhibited testicular failure (Garcia et al. 2014). Furthermore, in neonatal murine ovary, Notch ligands, *jagged1* and *jagged2*, were noted to be expressed in germ cells, whereas Notch receptors, *notch1* and *notch2*, were found to be expressed in pregranulosa cells (Johnson et al. 2001; Trombly et al. 2009), and blocking of Notch signal transduction was observed to affect ovarian development (Vanorny et al. 2014; Trombly et al. 2009; Manosalva et al. 2013; Chen et al. 2014; Feng et al. 2014). In our previous study, many differentially expressed genes were riched in Notch signaling pathway duringthe early stage of gonadal development (Jia et al. 2018), so we speculated that this pathway was involved in gonadal differentiation of carp.

As sex determination mechanism in fish is the most diversified among vertebrates, fish is an excellent model organism for studying the molecular mechanisms of sex determination and gonad development. Yellow River carp is the longest cultured and most widely domesticated fish species and has significant economic importance in China. As female carp could grow significantly faster than male carp after gonad differentiation (Gui 2007), mass production of female carp has always attracted increasing attention owing to its economic value. However, the genetic information for sex determination and gonad differentiation in carp remains unclear. In the present study, Yellow River carp *suh1* and *suh2* genes were verified and characterized, and N-[N-(3,5-difluorophenacetyl-L-alanyl)]-(S)-phenylglycine *t*-butyl ester (DAPT), which blocks Notch receptor proteolysis and is widely used in studying Notch signaling pathway, was employed for blocking the Notch signaling pathway in primordial gonad. The findings of this study could help in developing a new approach to regulate gonad development in Yellow River carp using Notch signaling pathway, as well as provide a novel method to study the molecular mechanisms of sex determination and gonad development in fish.

Materials and methods

Animals and sample collection

Yellow River carp were obtained from the Henan Academy of Fishery Science (Zhengzhou, Henan Province, China) and maintained at the Genetic Laboratory (Henan Normal University, Henan Province, China) in flow-through water tanks at 23 ± 2 °C under natural photoperiod for an initial acclimation period. Carp embryos were obtained by natural spawning and cultured in embryo medium following standard procedures. The staging of embryos was performed as described in a previous study (Chen 1960). The larvae were fed *Artemia nauplii* twice a day for the duration of the experiment, and no larvae died during the experiment. To study the expression profiles of *suh*, the embryos at blastula stage (2.5 h post-fertilization, hpf), gastrulae stage (7 hpf), neurula stage (13 hpf), tailbud stage (24 hpf), and hatching stage (3 days post-fertilization, dpf) were collected. Furthermore, tissues from heart, liver, spleen, kidney, forebrain, hindbrain, foregut, hindgut, gonads (ovary or testis), gill, scale, fin, eye, and skeletal muscle of adult male and female carp were obtained. Each sample was collected in triplicate. The collected embryo and tissue samples were immediately frozen in liquid nitrogen until RNA isolation. Before sample collection, the embryos and larvae were euthanized with 200 mg/L tricaine methanesulfonate (MS 222). This study was approved by the Committee of Laboratory Animal Experimentation at Henan Normal University (HNULSC101201). The DAPT (Sigma, USA) was dissolved in DMSO (Solarbio, China).

Sequence analysis of carp *suh* genes

Carp *suh1* and *suh2* sequences were obtained by RNA-Seq (Jia et al. 2018), and the open reading fragment

(ORF)sequences were verified by reverse transcription-PCR (RT-PCR) using the sequence-specific primers (Supplementary Table 1). The amplification conditions were as follows: 95 °C for 3 min, followed by 30 cycles of 94 °C for 60 s, 56 °C for 60 s, and 72 °C for 90 s, and a final extension at 72 °C for 10 min. The RT-PCR products were separated by electrophoresis on 1% agarose gel and visualized using a UV imaging system (Bio-Rad, USA). The resulting sequences were confirmed on the NCBI Blast Server. The deduced amino acid sequences of carp *suh1* and *suh2* were aligned with those from other vertebrates available in GenBank using ClustalW multiple alignment program software (http://www.ebi.ac.uk/Tools/msa/clustalo/). The molecular mass (MM) and isoelectric point of carp *suh1* and *suh2* were predicted by ProtParam tool (http://web.expasy.org/protparam/). A phylogenetic tree was constructed by neighbor-joining algorithms using MEGA6. The data of chromosome synteny of *suh1* and *suh2* among vertebrates were obtained from NCBI and carp genome sequences. Comparison of the flanking regions of *suh* among fish and mammals was performed with Dialign software from the Genomatix suite (http://www.genomatix.de/cgi-bin/dialign/dialign.pl). Bioinformatics analyses of the potential TF-binding sites within the 5′-regulatory region of carp *suh1* and *suh2* were performed using the online program MatInspector (http://www.genomatix.de/matinspector.html) (Gao et al. 2015).

Expression profiles of carp *suh* genes

The expression profiles of carp *suh1* and *suh2* were investigated with quantitative real-time PCR (qPCR) using Roche real-time PCR system (LightCycler96 Roche) with SYBR green fluorescent label. The primers of carp *suh1* and *suh2* were designed outside the conserved domains to prevent any non-specific amplification (Supplementary Table 1). The qPCR was performed under the following conditions: 95 °C for 10 s, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The $2^{-\Delta\Delta Ct}$ method was used to analyze the expression level. Each sample was amplified in triplicate to normalize the system and reduce pipetting error by using the standard curve method with *40S* (40S ribosomal protein S11) and *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) genes as reference genes (Zhang et al. 2016). Negative controls were included to confirm the absence of DNA contamination.

Effect of DAPT on gene expression in primordial gonad in vitro

The larvae at 40 days post-hatching (dph) were euthanized and their primordial gonads were isolated under a stereomicroscope. The gonads were washed thrice in PBS, transferred to a 24-well plate (BD Biosciences, USA), and maintained in a complex medium at 28 °C and 3% CO_2 in a humidified incubator. The complex medium contained 50% L15, 35% DMEM-high glucose, and 15% Ham's F-12 supplemented with 10% fetal bovine serum, 0.15 g/L sodium bicarbonate, 50 ng/mL mouse epidermal growth factor (EGF), and 0.01 mg/mL bovine insulin. The explants were cultured in parallel and divided into three groups: control group (without any added reagents to the medium), DMSO group (addition of 1% DMSO to the medium), and DAPT group (addition of 20 μM DAPT to the medium). Half of the medium was replaced with fresh medium every 48 h, and the experiments were repeated at least three times. Each group comprised six explants. After incubation for 1, 3, 5, and 7 days, the RNA of the explants was extracted using Total RNA Extraction Kit (RNAiso reagent, TaKaRa, Japan), and the cDNA was synthesized by using Prime Script Reverse Transcriptase (TaKaRa).

Long-term effect of DAPT on gonad development in larvae in vivo

A total of 300 larvae at 40 dph were randomly divided into three groups: control group in which the larvae were cultured in water without intervention, DMSO group in which the larvae were treated with 0.001‰ DMSO in water, and DAPT group in which the larvae were treated with 20 μM DAPT in water. The water was changed every 3 days, and after 30 days, the larvae were euthanized and dissected under a stereomicroscope. One side of the gonads was isolated, snap-frozen in liquid nitrogen, and stored at − 80 °C until further use. The other side of the gonads was pre-fixed in 4% PFA for 20 h at 4 °C, dehydrated through graded ethanol, embedded in paraffin, sectioned at 6 μm thickness, stained with hematoxylin-eosin, and observed under microscope to determine the stages of gonad and sex ratios. Three gonads of the same sex were selected for RNA extraction, and the cDNA was synthesized using Prime Script Reverse Transcriptase (TaKaRa).

Effect of DAPT on gene expression in primordial gonad in vivo

To confirm the effect of DAPT on Notch signaling pathway in the gonad of Yellow River carp, genes related to Notch signaling pathway (*suh1*, *suh2*, *notch1*, *notch2*, *notch3*, *jagged1*, *jagged2*, *aph1*, *pen2*, *her6*, *hey1*, and *hey2* genes) and gonad development (*amh*, *dax1*, *sf1*, *dmrt1*, *sox9a*, *nobox*, *foxl2*, *zp2*, *piwil*, *nanos*, *gdf9*, *figla*, *inhba*, and *inhbb* genes) were detected with qPCR. Some primers used were designed based on the known genes in NCBI, while the others were based on common carp genome database (http://www.carpbase.org/download_home.php). All the primer sequences used in this study are listed in Supplementary Table 1. The qPCR conditions were similar to those mentioned earlier, and $2^{-\Delta\Delta Ct}$ method was used to analyze the expression level. Each sample was amplified in triplicate to normalize the system, and all the reactions were independently repeated thrice to ensure reproducibility using the standard curve method with *40S* and *gapdh* as reference genes (Zhang et al. 2016). Negative controls were included to confirm the absence of DNA contamination.

Statistical methods

For each set of data, independent experiments were repeated at least three times, and the data were analyzed using SPSS 16.0 and presented as mean ± SD. Furthermore, multiple comparisons were performed, and all the parameters were tested for normality using unpaired Student's *t* test and analyzed using one-way ANOVA and Duncan's. Significant difference was accepted at $P < 0.05$.

Results

Sequence analysis of carp *suh* genes

Initially, the sequences of carp *suh1* and *suh2* were identified from the carp genomic data and found to be homologous to the *suh1* and *suh2* genes of other species, respectively, by BLAST search on NCBI. The full-length *suh1* cDNA was 6671-bp long, with a 1494-bp ORF, which encoded a protein of 497 amino acid residues (Supplementary Fig. 1). Comparison of genomic and cDNA sequences showed that *suh1* contained 11 exons and 10 introns, similar to the structure of zebrafish *suh1* and *suh2* (Supplementary Fig. 2). The estimated MM of the predicted amino acids was 55.64 kDa and the theoretical isoelectric point was 8.42. With regard to carp *suh2*, the full-length cDNA sequence was 4251 bp, with a 1473-bp ORF (Supplementary Fig. 3). Moreover, suh2 was found to comprise 11 exons and 10 introns (Supplementary Fig. 2), and the putative suh2 protein of 491 amino acids had an estimated MM of 54.60 kDa and a theoretical isoelectric point of 8.28.

BLAST and ClustalW analyses showed that the deduced amino acid sequences of carp suh1 shared higher identities with zebrafish suh1 (97.0%), and lower identities with human, chicken, alligator, and frog suh (88.3%-89.9%). When compared with suh2 homologs, carp suh2 presented total amino acid identities of 87.5% with carp suh1, and a much higher identity with zebrafish suh2 (95.1%) (Table 1). It must be noted that carp suh1 and suh2 had three domains that were highly conserved among species (Supplementary Fig. 4).

To evaluate the evolutionary relationships between the Yellow River Carp suh1/2 and other vertebrates suh, a phylogenetic tree was constructed based on the full-length amino acid sequences. The suh proteins were grouped into two distinct clades. The first clade was composed with most of the fish Suh, such as carp and zebrafish Suh2, *S. grahami.* Carp and zebrafish suh1, mammals, chicken, turtle and frog suh gene clustered into the other clade, and carp suh1 was first clustered with zebrafish suh1, then with the tetrapod suh gene (Fig. 1).

Chromosome synteny and genomic analysis of carp suh genes

The homologous relationship between carp suh1/2 and other species suh was determined by cross-species comparison of chromosome locations. Based on current *C. carpio* whole-genome sequencing data (http://www.carpbase.org/index.php), carp *suh1* and tandem genes (*chic* and *fip1*) were found to be repeated on LG2, whereas carp *suh2* was on LG36 and flanked by *cckar* and *itih5* (Fig. 2). The chromosome syntenic relationship of *suh1* and *suh12* was evolutionarily conserved between fish and mammals..

For promoter analysis, the transcriptional initiation site (ATG) was designated as + 1 and 2000-bp upstream flanking sequences of carp *suh1* and *suh2* were examined. The upstream flanking sequence of carp *suh1*

Table 1 Amino acid sequence percent identities of Yellow River carp Suh1 and Suh2 compared to other vertebrates Suhs proteins

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. C.carpio-Suh1	100%																	
2. D.rerio-Suh1	97.0%																	
3. C.carpio-Suh2	87.5%	87.9%																
4. D.rerio-Suh2	88.4%	89.4%	95.1%															
5. B.pectinirostris-Suh	89.3%	89.8%	91.3%	92.2%														
6. C.semilaevis-Suh	89.4%	90.2%	91.9%	92.4%	96.5%													
7. C.variegatus-Suh	88.8%	89.6%	90.8%	91.8%	94.3%	94.0%												
8. E.lucius-_Suh	88.7%	89.5%	90.5%	91.6%	93.6%	94.8%	91.8%											
9. L.bergylta-Suh	89.2%	90.2%	91.6%	92.7%	95.9%	98.2%	93.3%	94.5%										
10. S.partitus-Suh	89.8%	90.4%	92.2%	93.0%	96.7%	98.1%	93.7%	94.9%	99.0%									
11. L.crocea-Suh	89.4%	90.2%	92.2%	93.0%	96.3%	97.9%	93.5%	94.9%	98.8%	99.4%								
12. O.latipes-Suh	89.7%	90.1%	92.1%	92.6%	96.5%	97.5%	94.0%	95.0%	97.5%	98.4%	98.4%							
13. O.niloticus-Suh	89.6%	90.4%	90.9%	91.8%	95.7%	97.1%	92.8%	93.8%	98.0%	98.6%	98.4%	97.9%						
14. X.laevis-Suh1	89.7%	90.1%	89.7%	91.0%	91.7%	91.9%	92.2%	90.9%	91.8%	92.0%	92.0%	92.1%	91.5%					
15. G.gallus-SuhX1	88.3%	88.7%	89.7%	89.3%	90.3%	90.7%	90.7%	89.4%	89.9%	90.3%	90.3%	90.9%	89.9%	95.5%				
16. P.sinensis-Suh	88.5%	88.9%	89.9%	89.5%	90.5%	90.9%	90.7%	89.4%	90.1%	90.5%	90.5%	90.9%	90.1%	96.1%	97.8%			
17 .M.musculus-Suh	90.1%	90.6%	89.5%	90.3%	91.3%	91.7%	91.8%	90.1%	91.4%	91.5%	91.3%	91.5%	90.9%	96.7%	95.1%	95.7%		
18. H.sapiens-Suh	88.7%	89.1%	89.5%	89.5%	90.2%	90.5%	90.7%	89.0%	89.9%	90.3%	90.1%	90.2%	89.9%	95.3%	95.7%	96.3%	97.7%	

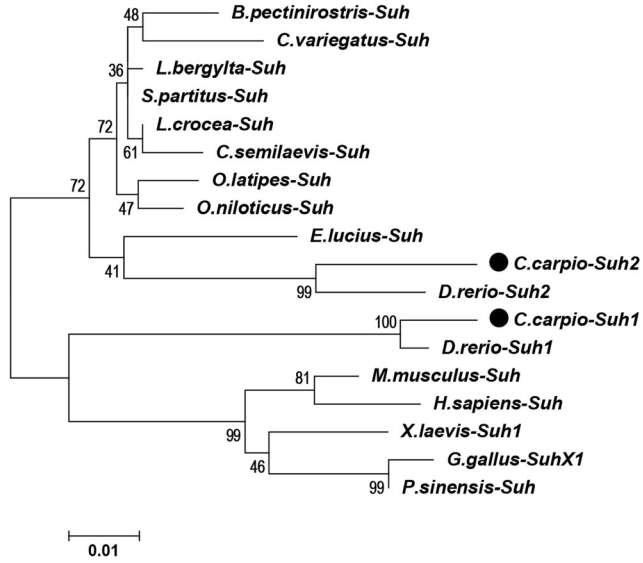

Fig. 1 Phylogenetic analysis of carp *suh1* and *suh2* in comparison with other representative vertebrates based on amino acid sequences. The tree was constructed by MEGA (version 6.0) using Poisson Correction distance based upon the neighbor-joining method with 1000 bootstrap replicates. The scale bar is 0.01. For GenBank accession numbers of sequences, see Supplementary Fig. 4

presented higher sequence conservation with zebrafish *suh1* at their 3′-terminus (Supplementary Fig. 5). Analysis using MatInspector predicted numerous essential TF-binding sites within the 5′-regulatory region, and those with a matrix score higher than 0.9 are illustrated in the schematic diagram presented in Fig. 3. Some of these TFs, including *sf1*, dmrt1, smad3, smad4, nobox, ar, *sox5*, *sox6*, *gata3*, and *foxl1*, were found to be involved in gonad differentiation and reproductive system development. Besides, some TFs closely related to various pluripotency or stem cell properties were identified, including *oct4*, *nanog*, and *foxi1a*. With regard to carp *suh1*, Nfκb signal pathway-related genes and liver-enriched and muscle-specific TFs such as *P65*, *foxa1 (hnf3α)*, *foxa2 (hnf3β)*, *hnf6*, and *mtbf* were detected. Besides, ubiquitous binding sites for *ap1*, *cebpb*, *usf*, *hsf2*, and *sp1* were also identified. The predicted TF-binding sites of carp *suh2* not only included *sf1*, *sox6*, and *nanog* genes, but also comprised *nobox*, *esr2*, and *sox30*, which were involved in gonad differentiation.

Expression pattern of carp *suh* genes

The expression pattern of carp *suh1* and *suh2* mRNA during embryonic development was analyzed with qPCR using *40S* and *gapdh* as reference genes. The highest level of *suh1* transcript was detected in the blastula stage. With the progression of embryonic development, the expression of *suh1* followed a declining trend, reaching the lowest level at hatching (Fig. 4).

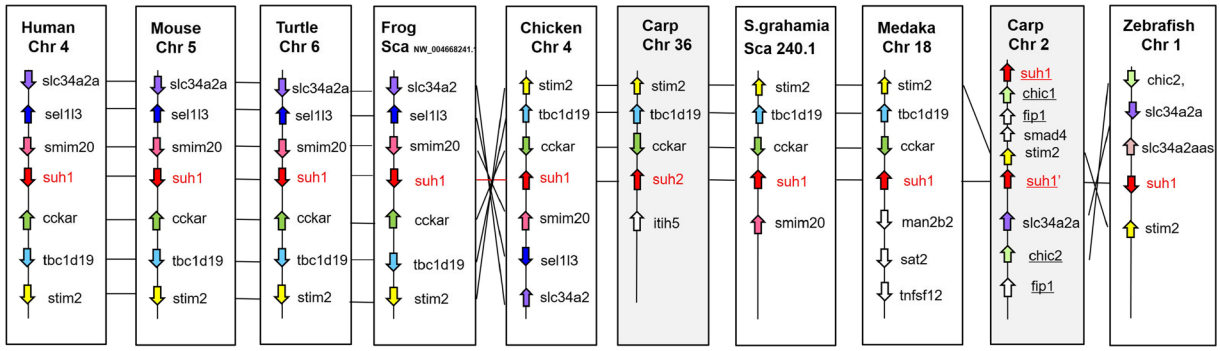

Fig. 2 Chromosome syntenic relationship of the fish *suh1* and *suh2* genes with their vertebrate orthologs. Conserved syntenies are shown for chromosomal segments containing *suh1*, *suh2*, and *suh*. Rectangles represent genes in chromosome/scaffold and arrows represent gene-coding direction. *Suh*, *smim20*, *cckar*, *tbc1d19*, *stim2*, and *slc34a2a* orthologs are shown in red, pink, green, light blue, yellow, and purple, respectively. Chr, chromosome; Sca, scaffold

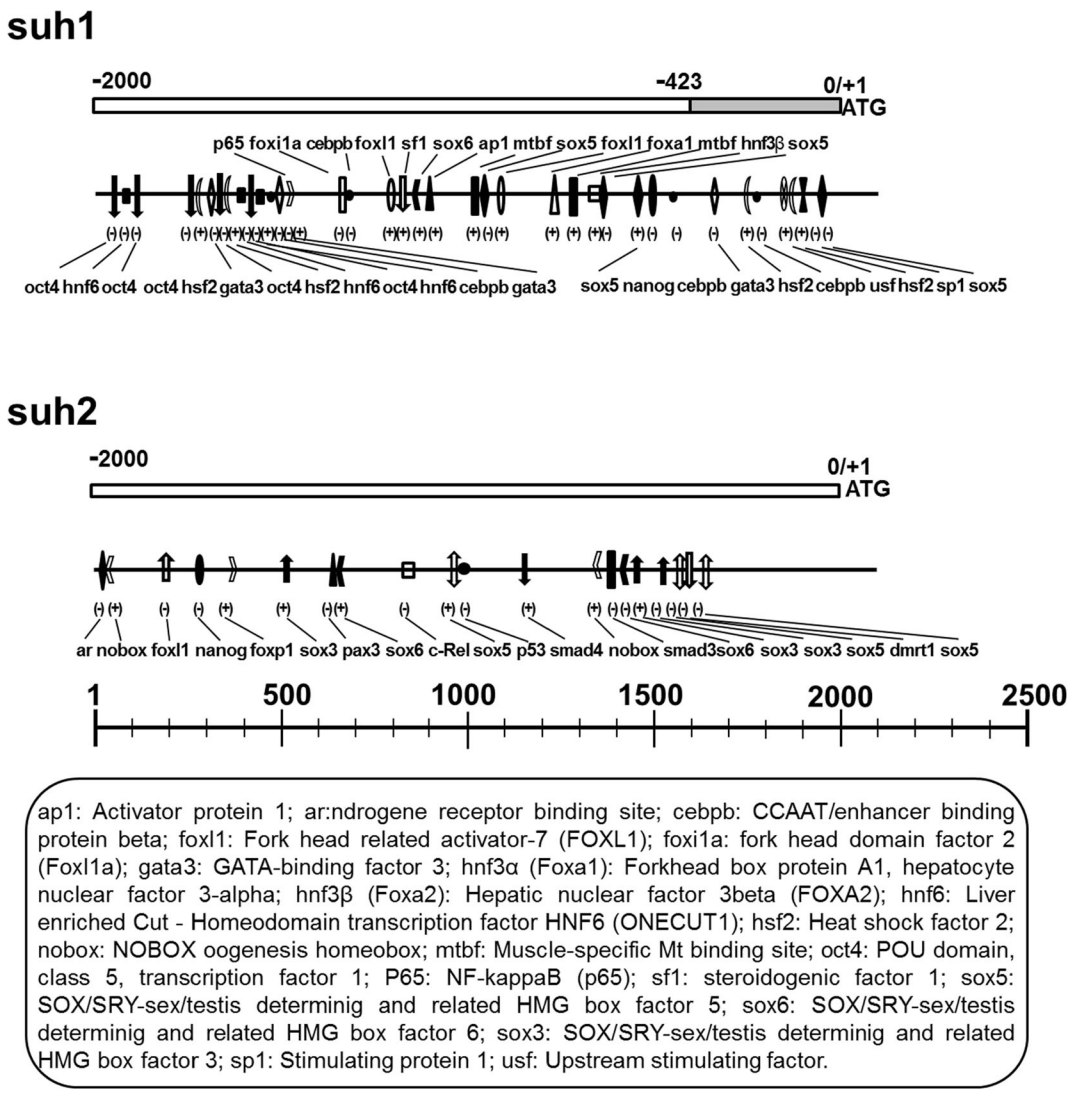

Fig. 3 Schematic diagram of putative regulatory motifs in the promoter of the *suh1* and *suh2* in Yellow River carp. The scale is given above and the full names of the potential TF binding sites are provided at the bottom. The plus-minus sign indicates the TF binding strand. The gray blocks represent the conserved upstream region among species (details can be seen in Supplementary Fig. 5). Transcriptional initiate site (ATG) is designated as + 1

Likewise, *suh2* exhibited a similar expression pattern, with the peak *suh2* expression noted in the early blastula stage. Besides, the amount of *suh1* transcript was lower than that of *suh2* transcript (Fig. 4).

Furthermore, expression analysis in various adult tissues revealed that *suh1* and *suh2* were predominantly expressed in the brain (Fig. 5). In particular, *suh1* transcript exhibited sexual dimorphism expression pattern in gonad, hindbrain, spleen, and gill ($P < 0.05$) (Fig. 5a), whereas *suh2* transcript was found at the highest level in the brain and at low level in fin and liver (Fig. 5b).

Histological characteristics of Yellow River carp gonads treated with DAPT

Histologically, primordial germ cells appeared in the primordial gonad of larvae at 40 dph (Fig. 6a). In the control group, the larvae completed sex differentiation at 70 dph, and the structural features of ovary or testis appeared (Fig. 6b, c). Furthermore, differentiated spermatogonium, primary spermatocyte, and spermatids were observed in the testis of the control group (Fig. 6c). In the DMSO group, the structural features of ovary

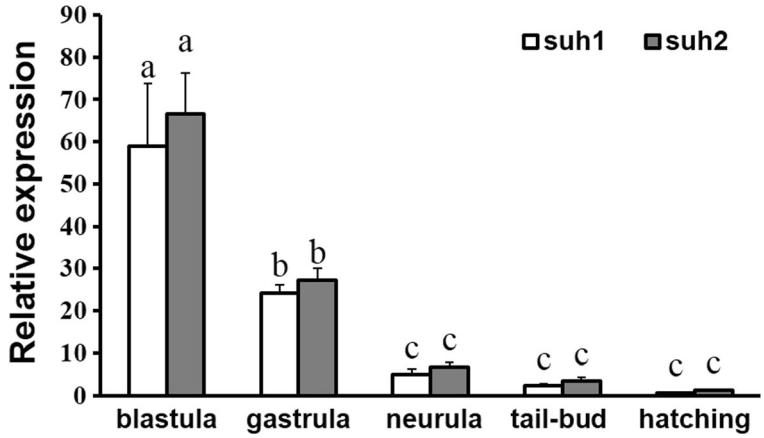

Fig. 4 Relative expression levels of carp *suh1* and *suh2* genes during embryonic development by qPCR analysis. Data are shown as mean ± SD ($n = 3$). Values with different letters indicate statistically significant difference ($P < 0.05$)

and testis were similar to those in the control group. In the DAPT group, although ovary differentiation was noted (Fig. 6d), testis development stagnation occurred, germ cells continued to undergo mitosis, and a cyst including two or four dividing germ cells and composed of somatic cells was observed. Moreover, no spermatogonium and primary spermatocyte appeared in the testis (Fig. 6e). At the end of the treatment (70 dph), the proportion of ovary in the control, DMSO, and DAPT groups was 49, 51, and 61%, respectively. These results revealed that DAPT might play a role in the inhibition of spermatogenesis at a crucial stage of gonadal development in Yellow River carp.

Effects of short-term DAPT exposure on gene expression in primordial gonad in vitro

The expression of Notch components and their target genes in primordial gonad was effectively inhibited by DAPT (Supplementary Fig. 6). *Suh1* were downregulated from day 1 to day 3 ($P < 0.05$). With regard to *aph1* and *pen2*, the target genes of DAPT, severe

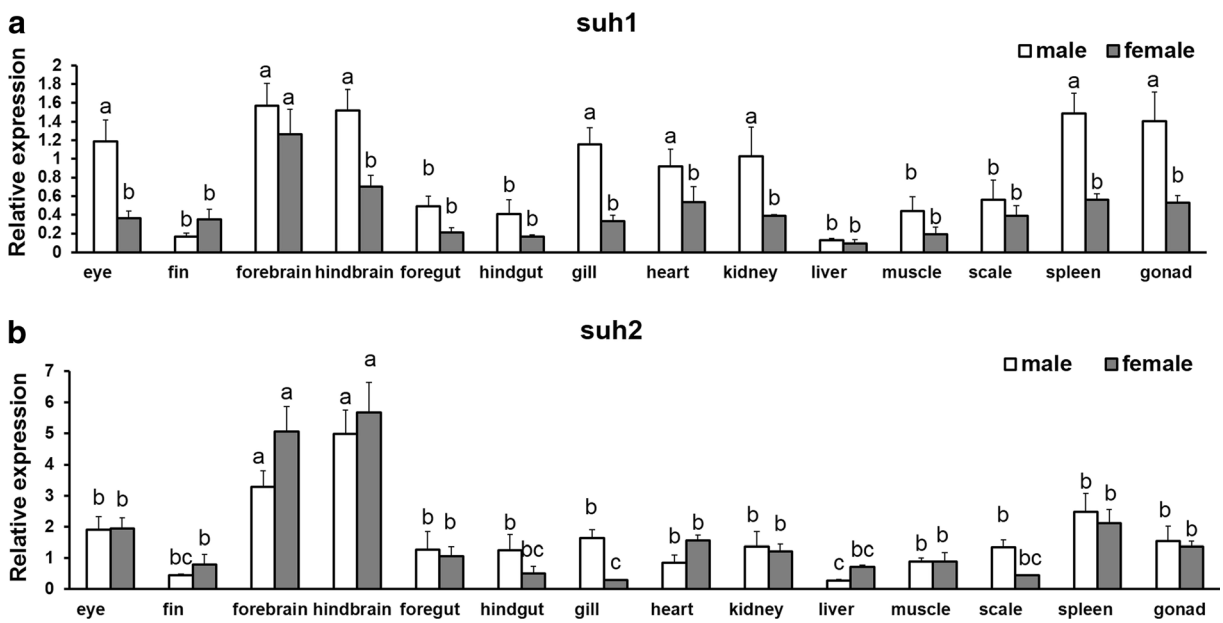

Fig. 5 Relative expression levels of carp *suh1* and *suh2* genes in different adult tissues by qPCR analysis. The relative expression variance is given as ratio (the amounts of carp *suh1* and *suh2* mRNA normalized to the values of the corresponding reference genes). Data are shown as mean ± SD ($n = 3$). Values with different letters indicate statistically significant difference ($P < 0.05$)

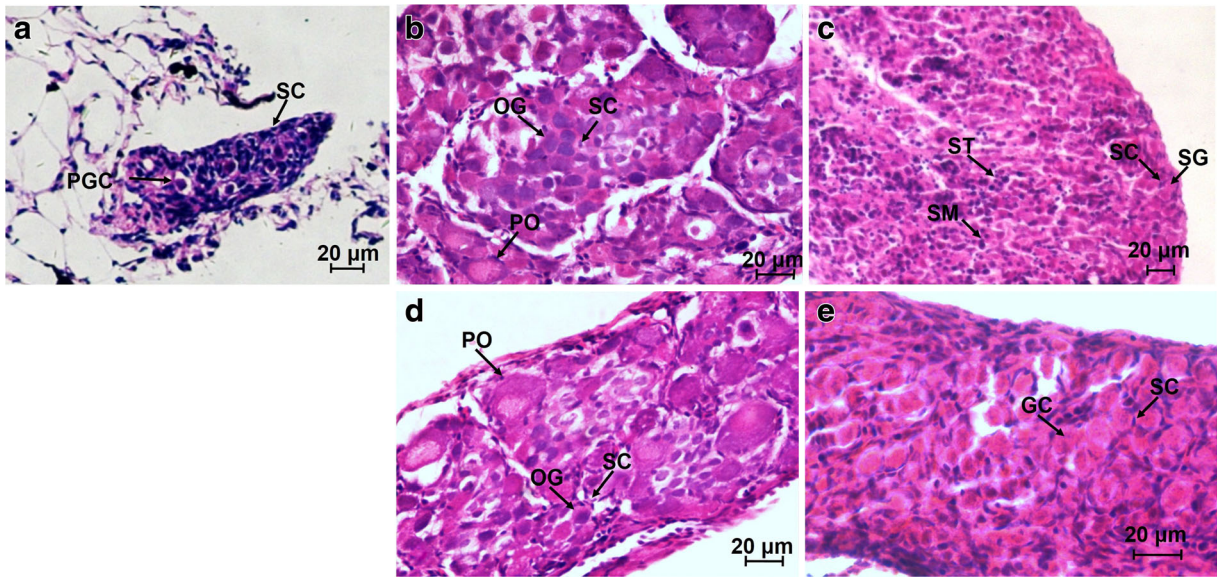

Fig. 6 Sections of Yellow River carp gonad. The same section was stained by hematoxylin and eosin (thickness, 6 μm). **a** Primordial gonad (40 dph). **b** Ovary of control group (70 dph). **c** Testis of control group (70 dph). **d** Ovary of DAPT group (70 dph). **e** Testis of DAPT group (70 dph). PGC: primordial germ cell, SC: somatic cell, OG: oogonium, PO: primary oocyte, SG: spermatogonium, SM: spermatocyte, ST: spermatid, GC: germ cell

downregulation of them were noted after DAPT exposure ($P < 0.05$).The Notch components, *notch1*, *notch2*, *notch3*, *jagged1*, and *jagged2*, were significantly downregulated after DAPT treatment ($P < 0.05$), and the downregulation of Notch target genes *her6* (*hes1*), *hey1*, and *hey2* persisted during the 7-day culture of primordial gonad with DAPT ($P < 0.05$, Supplementary Fig. 6).

It must be noted that *amh*, *dax1*, *sf1*, *dmr1t*, *sox9a*, *inhba*, and *inhbb* are related to the development of testis in fish. The expression of *sf1*, *dmrt1*, *sox9a*, *dax1*, and *inhbb* in the DAPT group was significantly lower, when compared with that in the control and DMSO groups ($P < 0.05$, Supplementary Fig. 7).

The genes *foxl2*, *cyp19a*, *nobox*, *zp2*, *piwil*, *nanos*, *gdf9*, and *figla* are critical for ovary development. The expression of *foxl2*, *piwil*, *gdf9*, *nobox*, and *zp2* in the DAPT group was significantly higher, when compared with that in the control and DMSO groups throughout the experiment period ($P < 0.05$). However, the expression of *cyp19a*, *cyp19b*, *nonos*, and *figla* in the DAPT group was not significantly different from that in the control and DMSO groups (Supplementary Fig. 7). These results suggested that DAPT/Notch signaling pathway affected ovary development in Yellow River carp by regulating the expression of *foxl2*, *piwil*, *gdf9*, *nobox*, and *zp2*, but not *cyp19a*, *cyp19b*, *nonos*, and *figla*.

Effects of long-term DAPT treatment on gene expression in primordial gonad in vivo

The expression of Notch genes was downregulated at various levels after 30 days of DAPT exposure. All Notch-related genes in the testis of the DAPT group were significantly downregulated, when compared with those in the testis of the control and DMSO groups ($P < 0.05$). In addition, most of thesegenes were also downregulated in the ovary of the DAPT group, and in particular, *suh1*, *pen2*, *jagged1*, *notch2*, and *her6* genes showed significant decrease in the ovary ($P < 0.05$, Supplementary Fig. 8).

In the control and DMSO groups, the gonads completed sex differentiation at 70 dph. Among the testis development-related genes, *amh*, *sf1* *dax1*, *sox9a*, *dmrt1*, *inhba*, and *inhbb* were significantly downregulated in the testis of the DAPT group, when compared with those in the control and DMSO groups ($P < 0.05$). With regard to gene expression in ovary, the expression of *sf1* and *inhba* was lower in the ovary of the DAPT group, when compared with that in the control and DMSO groups ($P < 0.05$, Supplementary Fig. 9). Furthermore, the expression of *foxl2*, *gdf9*, and *nobox* in the ovary of the DAPT group was significantly

increased, when compared with that in the control and DMSO groups ($P < 0.05$) (Supplementary Fig. 9).

Discussion

Sex determination in fish is extremely complex and can be affected by environmental factors and genetic system (Devlin and Nagahama 2002). As one of the most important signaling pathways, Notch signaling pathway plays an important role in regulating cell differentiation and gonad development in mammals (Trombly et al. 2009; Johnson et al. 2001; Chen et al. 2014); however, it is still unclear whether Notch signaling is involved in gonad development in carp. In the present study, the *suh* genes of carp were examined because they are crucial in Notch signaling pathway and sex dimorphism pattern in carp.

The results confirmed that *suh* is duplicated in Yellow River carp, similar to that observed in other fish species such as zebrafish (O'Brien et al. 2011). The notion that carp *suh1* and *suh2* are orthologs derived from a gene duplication event was also supported by their chromosomal localizations: different chromosomes contained duplicated genomic sequences; the structure, chromosome synteny, and genomic analyses of *suh1* and *suh2* suggested that they are conserved.

Although *suh* gene is the main effector in Notch pathway, its functions and TF-binding sites have not yet been reported. The present study examined the TF-binding sites of carp *suh1* and *suh2*, which might participate in the regulation of gene expression and function, and is the first to predict the TF-binding sites of carp *suh* genes (Supplementary Tables 2 and 3). Some TF-binding sites relevant to gonad differentiation, including *sf1*, *sox5*, *sox6*, *nobox*, *esr2*, and *GATA3*, in vertebrates (Espinosa et al. 2015; Mu et al. 2013; Connor et al. 1995; Denny et al. 1992) may interact with carp *suh1* and *suh2* to regulate their expression in gonad development in carp.

The *suh* gene is a Notch transcriptional mediator, and previous studies have suggested that this gene is critical in Notch pathway and early processes in embryogenesis and testis development (Zhang et al. 2014; Garcia et al. 2014). However, to date, there are only few reports on teleost *suh* (O'Brien et al. 2011). The sexual dimorphism expression pattern of carp s*uh1* in gonad observed in the present study indicated its involvement in gonad differentiation. Furthermore, DAPT has been observed to block the Notch signal transduction in the ovary of mammals in vitro and inhibit gonad development (Chen et al. 2014) and has been widely used to study the development of tissues and organs by blocking the Notch signaling pathway (Chen et al. 2014; Feng et al. 2014; Zhang et al. 2011). However, the molecular mechanisms of gonad development in carp are still unclear (Chen et al. 2015; Gui 2007), and the signal pathways in the carp gonad, which regulate gonad development and sex differentiation, have not been extensively studied. In the present study, after short-term treatment with DAPT in vitro, the expression of Notch genes, such as *suh1*, *notch1*, *notch2*, *notch3*, *jagged1*, *jagged2*, *apha1*, and *pen2*, was significantly downregulated in primordial gonad. Moreover, following long-term DAPT exposure, *suh1*, *suh2*, and other Notch-related genes were significantly downregulated in the testis of the DAPT group, when compared with that in the control and DMSO groups ($P < 0.05$). In addition, *pen2*, *notch2*, *jagged1*, and *her6* in the ovary of the DAPT group were also significantly downregulated ($P < 0.05$). These results revealed that Notch signaling was suppressed by DAPT during the early development of gonad in Yellow River carp.

It has been reported that *amh*, *dmrt1*, *sox9a*, *sf1*, *dax1*, *inhba*, and *inhbb* play roles in testis development in fish (Zhou et al. 2014l Rodriguez-Mari et al. 2005; Hattori et al. 2012; Herpin and Schartl 2011; Herpin et al. 2013; Hornung et al. 2007; Tada et al. 2002), and the expression of these genes was detected with qPCR in the present study. Furthermore, these genes were downregulated, especially in testis, after DAPT treatment both in vitro and in vivo ($P < 0.05$). Besides, DAPT delayed the development of testis, germ cells continued to undergo mitosis in cyst, and spermatogonium and primary spermatocyte did not appear in the testis at 70 dph. These findings suggest that transcription factors *amh*, *dax1*, *dmrt1*, *sox9a* are sensitive to DAPT, and their decreased expression in testis could affect the development of testis. Therefore, it can be assumed that DAPT could suppress testis differentiation by regulating the expression of *amh*, *dax1*, *dmrt1*, *sox9a*, and *sf1*.

The *foxl2*, *figla*, *gdf9*, *piwil*, *zp2*, *nanos*, and *nobox* genes are critical for the development of ovary (Zhou et al. 2014; Herpin et al. 2013; Liu and Ge 2007; Chen et al. 2015; Draper et al. 2007; Rajkovic et al. 2004; Huang et al. 2014; Jia et al. 2016). In the present study, the expression pattern of these genes was enhanced in the ovary, when compared with that in the testis, and

their levels of expression varied in gonads subjected to DAPT treatment. Previous studies had shown that primordial follicle formation was regulated by Notch pathway by affecting the expression of *nobox* gene (Chen et al. 2014). In the present study, the expression of *foxl2*, *gdf9*, and *nobox* was higher in the primordial gonad after DAPT treated in vitro, indicating that DAPT could increase the expression of *foxl2*, *gdf9*, and *nobox*. Similar results were noted in ovary following long-term DAPT exposure. Taken together, these results revealed that DAPT participates in ovary differentiation by regulating the expression of *foxl2*, *gdf9*, and *nobox*. It has been reported that *piwil* gene is crucial for the differentiation and development of germ cells (Zhou et al. 2014; Zhou et al. 2012). After long-term DAPT exposure, the differentiation and meiosis of germ cells in testis were suppressed by downregulated expression of *piwil*. The testis section of the DAPT group revealed that the germ cell differentiation was not initiated and the cells remained in the germ cell syncytia, whereas spermatids already appeared in the testis of the control group. In contrast, higher expression of *piwil* in ovary helped in the differentiation of oocytes, although its regulation mechanism is not clear and requires further investigation. Moreover, the higher proportions of ovary at 70 dph suggest that the increased expression of *foxl2*, *gdf9*, *nobox*, and *piwil* positively regulates the early development of ovary. Sex differentiation is a complex process, including changes in early genetic levels and subsequent morphological changes. A*mh*, *dax1*, *dmrt1*, *sox9a*, *foxl2*, *gdf9*, *nobox*, and *piwil* are crucial transcription factors during early gonad differentiation. The expression of them was changed significantly after DAPT treatment IN primordial gonad suggested notch signal/DAPT should be involved in the early differentiation of the gonads by regulating these upstream genes in the process of gonad differentiation.

Conclusions

In this study, the structure of Yellow River carp *suh1* and *suh2* genes was described and their expression profiles during embryo development as well as in adult tissues were examined. In addition, the potential regulatory TF-binding sites in the upstream flanking sequences of *suh1* and *suh2* were analyzed. The sexual dimorphism expression pattern of s*uh1* suggested its potential functions in the regulation of gonadogenesis in Yellow River carp. Chromosome synteny analysis indicated that the carp *suh1* and *suh2* are highly conserved during evolution. These results could help in further understanding of the functions of *suh* gene in teleost fish. In our previous study, results showed that DAPT could affect the sex ratio during the early development of gonads in carp. The mechanism should be related to the notch signal/DAPT is involved in early gonadal differentiation by regulating the expression of transcription factors upstream of the genetic regulation.

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Compliance with ethical standards

This study was approved by the Committee of Laboratory Animal Experimentation at Henan Normal University (HNULSC101201).

Conflict of interests The authors declare that they have no conflict of interest.

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