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Cloning, genomic structure and expression analysis of *ubc9* in the course of development in the half-smooth tongue sole (*Cynoglossus semilaevis*)

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ABSTRACT

The small ubiquitin-like modifier (SUMO) pathway is an essential biological process in eukaryote, and Ubc9 is an important E2 conjugating enzyme (UBE2) for SUMO pathway and plays a critical role in cellular differentiation, development and sex modification in various species. However, the relationship between Ubc9 and sex modification and development in fish remains elusive. To elucidate the impact of Ubc9 on sex modification and development, the full length of the cDNA and genomic sequence was cloned from half-smooth tongue sole, *Cynoglossus semilaevis*. Real-time quantitative RT-PCR demonstrated that *ubc9* was ubiquitously expressed in different tissues, and the expression levels varied in the different stages of embryonic and gonadal development. In addition, the expression level was significantly higher in the temperature-treated females than the normal females and males. Moreover, the PET-32-Ubc9 plasmid was constructed and the recombinant protein was expressed in *Escherichia coli*. Follistatin gene expression was initially up-regulated and FSE genes (*cyp19a1a, ctnnb1, foxl2*) were initially down-regulated after the injection of Ubc9 protein, prior to 96 h eventually recovered to normal levels. Taken together, the results show that Ubc9 is involved in embryogenesis, gametogenesis and sex modification, and exerts an effect on gene expression.

1. Introduction

Half-smooth tongue sole (*Cynoglossus semilaevis*) is an important economic marine fish species in China because of its agreeable taste. Half-smooth tongue sole shows significantly sexual dimorphism in growth and the female individual grows faster than the male. Male and neo-male at a small size devalued the commercial production. Artificial gynogenesis was performed to alleviate the issue of more male in fry, but it had achieved little success (Chen et al., 2009). It is necessary to elucidate the mechanisms of sexual growth dimorphism. However, the mechanism of sexual growth dimorphism remains unknown. According to the early studies, we learn that there is a significant correlation between development and growth. For instance, Australian longfinned eel (*Anguilla reinhardtii*) gonadal development stages were correlated with body size in both sexes (Walsh et al., 2003). Atlantic halibut males exhibited slow growth comparing with-females due to precocious sexual maturation (Jákupsstovu and Haug, 1988). Therefore, the investigation of the molecular mechanisms of embryogenesis, gametogenesis and sex reversal may provide a useful way to predict sex reversal and early maturation, and thus serve as a useful tool for increasing the production of this species.

Ubiquitin is a highly-conserved regulatory protein that is ubiquitously expressed in eukaryotes. Small ubiquitin-like modifiers (SUMO) comprise a family of proteins which covalently attach to target proteins. SUMO proteins are made up of three types, UBE1, UBE2 and UBE3. Sumoylation is comprised of a three-step pathway: a precursor SUMO protein is modified by cysteine proteases that cleave the C-terminus of the precursor to form the mature SUMO protein. The mature protein can be activated by the SUMO UBE1 enzyme. The SUMO UBE2 enzyme (Ubc9) conjugates the mature protein to target proteins. The SUMO UBE3 ligase transfers the activated ubiquitin/SUMO from UBE2 to the substrate. Although SUMO conjugation can be carried out by Ubc9 without UBE3 ligase in certain cases, both the efficiency and specificity are enhanced by SUMO UBE3 ligase. The SUMO pathway has a number of effects, such as DNA repair, gene transcription, cell cycle regulation, tumor suppression and chromosome stabilization, and it also plays a key role in the control of cell growth (Schmidt and Muller, 2003; Tago et al., 2005; Artus et al., 2006; Terada and Furukawa, 2010; Yuan et al., 2010; Prudden et al., 2011; Qin et al., 2011; Suda et al., 2011).

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Ubc9 is a member of the ubiquitin-conjugating enzyme family and has a highly conserved catalytic site. Studies have demonstrated that Ubc9 plays an important role in cell cycle regulation and DNA repair (Seufert et al., 1995; Mo et al., 2004). In the embryogenesis stage, the loss of Ubc9 in *Drosophila melanogaster* results in an anterior–posterior dysregulation of morphogen bicoid and leads to a patterning defect (Epps and Tanda, 1998). In *D. melanogaster* adult, Ubc9 is involved in the development and function of gonads (Hashiyama et al., 2009; Zhang et al., 2010). The deficiency of Ubc9 in *Saccharomyces cerevisiae* is lethal, and it mediated degradation of mitotic cyclins in mitosis (Seufert et al., 1995). Recent studies suggested that Ubc9 may play an important role in sex modification. For instance, in mice Ubc9 interacts with and modulates the transcriptional activity of Foxl2 (Marongiu et al., 2010). Furthermore, Ubc9 weakens the activity of the Steroidogenic Acute Regulatory (StAR) promoter and the mutation of lysine 25 to arginine leads to the loss of transcriptional repressor activity of Foxl2 (Kuo et al., 2009).

In fish, several studies have reported on the activities of Ubc9 in *Danio rerio*, such as mitosis, cell survival, interaction with a nuclear localization signal and the early development of zebra fish (Kurtzman and Schechter, 2001; Nowak and Hammerschmidt, 2006; Yuan et al., 2010). However, the reports on Ubc9 in marine fish have thus far been scant. In this study, we first cloned the complete cDNA and DNA sequences in *C. semilaevis* and assessed the expression of *ubc9* in different tissues, as well as different stages of embryonic and gonadal development. To investigate the impact of Ubc9 on development and sex modification, we constructed the recombinant plasmid PET-32a-Ubc9 and the recombinant protein RUBC9 was expressed in *Escherichia coli*. Subsequently, the protein was purified and injected into the ovary of the female, after which the expression of female strongly expressed genes (FSE) (aromatase cytochrome P450A (*cyp19a1a*), forkhead box L2 (*foxl2*) and Beta-catenin1 (*ctnnb1*)) and the Follistatin (*fst*) gene was detected by RT-PCR.

2. Materials and methods

2.1. Tissue and embryo collection

C. semilaevis were obtained from the Haiyang High-Tech Experimental Base (Haiyang, Shandong Province, China). Twelve tissues (heart, liver, gill, skin, blood, kidney, intestine, brain, spleen, muscle, pituitary and gonad) were collected from one-year old *C. semilaevis*, and quick-frozen in liquid nitrogen until use. The gonad from one side of fish at different stages was collected and stored in liquid nitrogen until use. In addition, the gonad of the other side was collected in Bouin's fixative for gonadal histology. Simultaneously, to determine the genetic sex, the fin was collected in 100% ethanol for DNA extraction. The embryos at different stages were treated in phosphate buffer saline (PBS; 10 mM, pH 7.4) before being frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. The *E. coli strain* Top10 was used as a host for propagation of the recombinant forms of the plasmids PET-32a and PET-32a-Ubc9. *E. coli* BL21 was used for the expression of recombinant proteins. Plasmid PET-32a was maintained in the lab.

2.2. Induction of sex reversal in C. semilaevis fry

The induction of sex reversal in *C. semilaevis* fry by temperature treatment was performed as described (Deng et al., 2009). On day 28 after hatching, the fry were cultured in tanks at a temperature of 28 °C until 100 days and then grown into adults at a natural water temperature (22 °C). Three repeats were performed in the temperature-treated group and three in the untreated group, and each group contained 200 tongue soles.

2.3. Physiological sex and genetic sex identification

The gonad histology was carried out as described (Chen et al., 2008a). Genetic sex identification of *C. semilaevis* was determined subsequently (Chen et al., 2007). The genomic DNA from each individual was extracted using a phenol chloroform protocol with RNase I (EC: 3.1.4.22) treatment. The female-specific primers were CseF382F and CseF382R (Table 1). A 291 bp band was related to the genetic female, while the genetic male did not have this female-specific band. In addition, the fish that were physiologically male and genetically female were deemed to be neo-male.

2.4. Cloning of C. semilaevis partial ubc9 cDNA

RNA extraction and cDNA reverse transcription were performed according to an established procedure (Ji et al., 2011). One pair of degenerate primers, Ubc9-hf1 and Ubc9-hr1, was designed following the sequence of *ubc9* conserved across different species (Table 1) to clone the partial fragment of *ubc9* PCR amplification was performed in a typical, i.e. the amplification program was 94 °C for 5 min, followed by 32 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min and a final 5 min at 72 °C.

2.5. 5'-and 3'-RACE amplification of ubc9

According to the partial *ubc9* cDNA sequence, two pairs of RACE-specific primers (Ubc9-5'GSP and Ubc9-3'GSP) were designed for the 5' end and 3' end amplification, respectively. Using a BD SMART™ RACE cDNA Amplification kit, both 5'-RACE and 3'-RACE were carried out according to the manufacturer's instructions. PCR performed as follows: 5 cycles of 94 °C for 30 s and 72 °C for 3 min, 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 3 min, then 27 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. All of the amplification products were run on 1% agarose gel using the D2000 marker (Takara, Dalian, China). Bands of the expected size were excised from the gel and purified with a Tiangen gel extraction kit (Tiangen, Beijing). The purified fragment was cloned into a PMD-18-T vector, and conveyed into *E. coli* TOP10, and sequenced. The *ubc9* cDNA sequence was analyzed by bioinformatics and one pair of primers (Ubc9-gf and Ubc9-gr) was designed at each side for genomic amplification (Table 1).

2.6. Bioinformatics

Sequences were analyzed based on nucleotide and protein databases using the online website (http://www.ncbi.nlm.nih.gov/BLAST/). The coding sequence (CDS) was predicted by the online website (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). The ubiquitin domain was analyzed by means of an NCBI conserved domain search (http://www.ncbi.nlm.nih.gov/structure). The protein molecular weight was deduced using the website (http://www.bio-soft.net/sms/index.html). The signal peptides were predicted with Signalp3.0 (http://www.cbs.dtu.dk/services/SignalP/). Multiple alignments of amino acid (AA) sequences were analyzed by Clustal W. Phylogenetic relationships were deduced using Mega 4 software.

2.7. Plasmid construction, protein expression and purification

One pair of primers was designed to construct the plasmid (Table 1). The template was the cDNA reversed from the RNA in the testis. PCR amplification was initiated at 94 °C for 4 min, 32 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The constructed plasmid (PET-32a-Ubc9) was transformed into *E. coli* strain BL21 after the plasmid had been sequenced. The transformants were grown in 5 mL LB containing 100 mg/mL ampicillin at 37 °C to an OD_{600} of 0.6 and then

Table 1
Primers and their sequences used in this study.

Primer	Primer sequences (5'to3')	Utilizations
CseF382F	5'ATTCACTGACCCCTGAGAGC3',	Genetic sex determination
CseF382R	5'TGGCACCATCATTGTAAAACTA3'	Genetic sex determination
Ubc9-hf1	CAGACTGTCTCAGGAGCGCAA	Partial fragment clone
Ubc9-hr1	GGGCAAACTTCTTGGCCTG	Partial fragment clone
Ubc9-5'GSP	CACTGTGCCTGACGGATAGACATTTGGGT	5'region clone
Ubc9-3'GSP	AGGAACCTTATGGGAGGGAGGGCTTTACA	3'region clone
UPM-long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	5' or 3' RACE
UPM-short	CTAATACGACTCACTATAGGGC	5' or 3' RACE
Ubc9-gf	ATGTCTGGCATTGCTCTTAGC	Genome of UBC9
Ubc9-gr	CTATGTGGGAGCAAACTTCTTG	Genome of UBC9
Ubc9-RT-f	GTTTGATGGTGATGGCTGGTC	RT-PCR or quantitative RT-PCR
Ubc9-RT-r	TTATGGGAGGGAGGGCTTTAC	RT-PCR or quantitative RT-PCR
β-Actin-f	CCAACAGGGAAAAGATGACC	Internal control
β-Actin-r	TTCTCCTTGATGTCACGCAC	Internal control
PET-32-Ubc9a	CGGGAATTCTGTGGGAGCAAACTTCTTGG	Construction of plasmid
PET-32-Ubc9s	GGCGATATCATGTCTGGCATTGCTCTTAGC	Construction of plasmid
Foxl2-a	GAGAGGAAGGGCAACTACTGGA	RT-PCR or quantitative RT-PCR
Foxl2-s	TGGTTGGAAGTGCGTGGG	RT-PCR or quantitative RT-PCR
CYP19A1A-a	GGTGAGGATGTGACCCAGTGT	RT-PCR or quantitative RT-PCR
CYP19A1A-s	TGAGACACCAACTCCACCCG	RT-PCR or quantitative RT-PCR
FST-a	AGGAGGAAGAACTTGGGATTTGT	RT-PCR or quantitative RT-PCR
FST-s	GCACTCGTCCCTGCTCATTC	RT-PCR or quantitative RT-PCR
Ctnnb1-a	ATAATGCAGTCTCACGGCGTT	RT-PCR or quantitative RT-PCR
Ctnnb1-s	TCTGTCAGGTTGGCGGTATTG	RT-PCR or quantitative RT-PCR

isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mmol/L. Bacteria were grown for an additional 6 h at 28 °C, centrifuged for 5 min at 17,508 *g* in a chilled microcentrifuge and the pellets were resuspended in 200 mL PBS (10 mM, pH 7.4), washed twice in the same solution and centrifuged again. The resultant pellet was resuspended in 100 mL of Laemmli's sample buffer (63 mM Tris HCl, 10% glycerol 2% SDS, 0.0025% bromophenol blue, 2.5% beta-mercaptoethanol, pH 6.8) (Laemmli, 1970). Samples were heated at 100 °C for 5 min, chilled on ice and then centrifuged briefly. An aliquot of 10 mL was analyzed by 12% SDS-PAGE. A non-induced control culture was analyzed in parallel.

The protein was purified from the bacteria (250 mL) after having been induced for 6 h. Centrifugation was performed at 8000 g for 10 min at 4 °C, then the wet mass of 1 g *E. coli* cell pellet was resuspended in a 20 mL volume of column buffer (20 mM Na_3PO_4, 0.5 M NaCl, 30 mM imidazole, pH 7.4) and 20 U DNase I(EC:3.1.21.1), 4 mg lysozyme (EC: 3.2.1.17), and then 1 mM PMSF was added to digest the bacteria. The cell debris was removed by centrifugation at 10,000 *g* for 10 min at 4 °C after intermittent sonication at 200 W for 10 min. The supernatant was collected and filtered with a 0.45 μm filter (Millipore). The supernatant, after filtration, was loaded on a column buffer-equilibrated column (1 mL, General Electric, USA) at a flow rate of 1 mL/min. Then the fusion protein PET-32a-Ubc9 was eluted with 10 mL elution buffer (20 mM Na_3PO_4, 0.5 M NaCl, 500 mM imidazole, pH 7.4)

2.8. Protein injection

The protein density was determined with a Bradford protein assay kit (Tiangen). One year old female fish (n = 39) were collected for protein injection. The ovary was checked under strong light. Twenty-one females were injected at ovary with 150 μL (1 mg/mL) purified protein in the experimental group and the same dosage of heat-denatured protein (100 °C for 5 min) was injected into another 18 females in the control group. Gonads were collected at seven time points (0 h, 6 h, 12 h, 24 h, 48 h, 72 h, 96 h) for RNA isolation, and on each occasion three individuals were sacrificed in each group.

2.9. Real-time quantitative PCR

The mRNA expression of *ubc9* was performed independently in triplicate and at least three samples of different tissues and different stages of the embryo and gonads were processed by real-time quantitative PCR (RT-QPCR). QPCR was performed in reaction systems (20 mL) in a 7500ABI real-time PCR system (Applied Biosystems, Foster City, CA, USA) The reaction was carried out as described in the manufacturer's manual using 10 mL SYBR Premix Ex Taq (2X), 0.4 mL of Ubc9-RT-f and Ubc9-RT-r, 1 mL cDNA and 0.4 mL ROX reference dye. The PCR amplification procedure was as follows: 95 °C for 10 s, 40 cycles of 95 °C for 5 s, 60 °C for 34 s, followed by disassociation curve analysis to determine target specificity. β-Actin was used as the internal control. The concentration of cDNA in each sample was reflected by the crossing point (Ct) values, which were compared using the relative quantification method and 7500 system SDS software (Applied Biosystems).

2.10. Statistical analysis

The RT-QPCR was repeated for three times, and the data were log-transformed. Then the treated data were analyzed by one-way ANOVA followed by Duncan multiple comparison tests using SPSS 17.0 (IBM, New York, NY, USA). Significance was set at $p < 0.05$.

3. Results

3.1. cDNA and genomic sequences of Ubc9

Based on the degenerate primers Ubc9-hf1 and Ubc9-hr1, the expected 450 bp fragment was obtained. Based on the partial fragment, the two specific primers 5'GSP and 3'GSP were designed and used for 5'-RACE and 3'-RACE. Two distinct bands of the predicted sizes of 420 bp and 1463 bp were produced in 5'-RACE and 3'-RACE, respectively. The fragments were sub-cloned and sequenced. Using vector NTI Advance 11 software, the full-length *ubc9* cDNA sequences of 1863 bp was obtained (Genbank accession No. JQ765845), including a 296 bp 5' terminal UTR, 477 bp in the open reading frame (ORF)

and a 1090 bp 3' terminal UTR containing a polyadenylation signal (AATAAA)(Fig. 1A). The ORF encodes a protein with 158 AA residues, with a predicted molecular weight of 18.09 kDa and no signal peptide. Based on the bioinformatics analysis, Ubc9 was predicted to have one cysteine residue (Cys93) as an active site. The Ubc domain contained both ubiquitin-like thioester intermediate interaction residues and UBE3 interaction residues (Fig. 1). The full length of the 2976 bp *ubc9* genomic DNA was obtained (Fig. 1A), with six exons and five introns, which is similar to the *ubc9* gene of *Takifugu rubripes*. In the third intron, a 64 bp of microsatellite sequence was predicted to have $(TA)_{32}$.

3.2. Sequence alignment and phylogenetic analysis of the ubc9 gene

Alignment of the cDNA sequence of the half-smooth tongue sole *ubc9* gene by BLASTx search in other species showed that the CDS of *C. semilaevis* displays a high degree of identity with many other fish. For example, the CDS of *ubc9* shared 88% identity with *Oreochromis niloticus* (XM_003443133.1), 88% identity with *Larimichthys crocea* (FJ195328.1), 87% identity with *Osmerus mordax* (BT074509.1) and 87% identity with *Salmo salar* (BT125220.1). Furthermore, the deduced Ubc9 AA sequence had completely identical to the ubiquitin-conjugating enzyme UBE2 of *O. niloticus* (XP_003443181.1) and shared 96% with *Homo sapiens* (NP_003336.1), 85% with *Scylla paramamosain* (ACJ03792.1) and shared 76% with *Caenorhabditis remanei* (XP_003108197.1). These analyses demonstrated that the CDS of *ubc9* had a high degree of identity in the different species. Following the highly conserved Ubc domain, the UBE3 interaction residues and active site cysteine residue were then predicted (Fig. 1A). The ubiquitin-like thioester intermediate interaction residues region was also analyzed (Fig. 1A). A phylogenetic tree was generated by Mega 4 software with the sequence of the Ubc9 proteins obtained from NCBI. The tree showed that all of the vertebrata had a high homology and close relationship. The fish *O. niloticus*, *Oryzias latipes*, *L. crocea* and *C. semilaevis* were grouped into one set of homologues and *D. rerio*, *Oncorhynchus mykiss*, *H. sapiens* to be grouped into another (Fig. 2). Simultaneously, insects and crustaceans clustered in one branch (Fig. 2). The results of bioinformatics analysis showed that Ubc9 is the gene of an ubiquitin-conjugating enzyme.

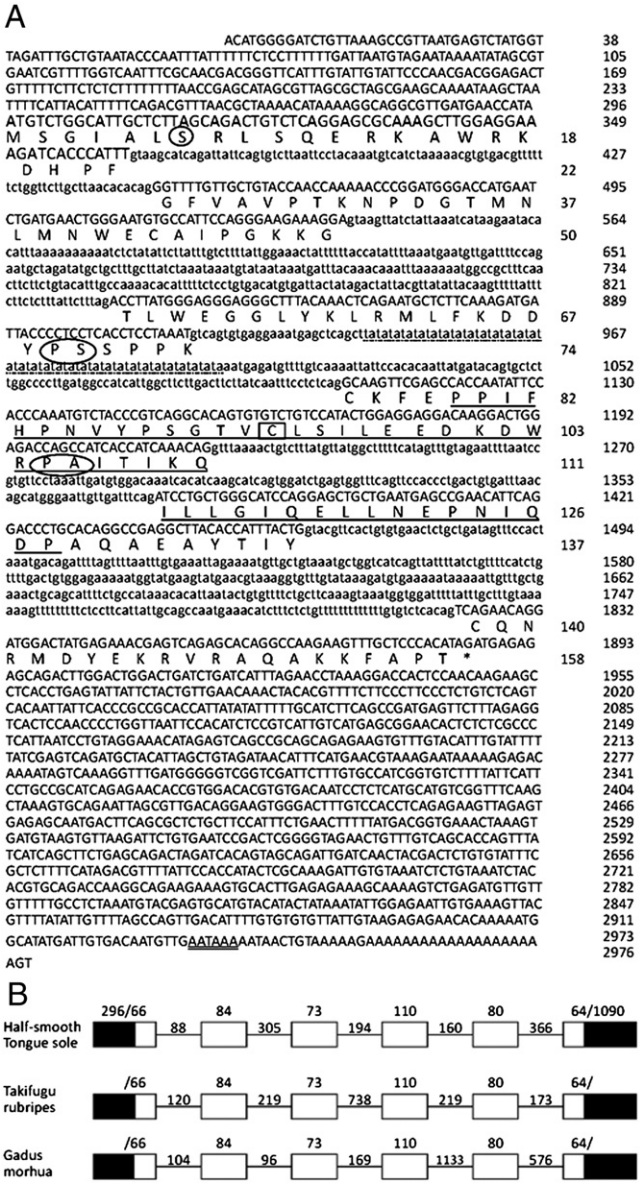

Fig. 1. Genomic sequences (A) and schematic illustration (B) of half-smooth tongue sole *ubc9* gene. Exons are in uppercase and introns are in lowercase. The stop codon is indicated by an asterisk. The termination signals (AATAAA) in the 3'-untranslated region (UTR) are underlined with ====. Ubiquitin-like thioester intermediate interaction residues region is underlined. Ligase (UBE3) interaction residues are marked by ovals. The box marked the active site cysteine residue of amino acid sequence of UBC domain. Microsatellite sequences are marked by — —.B. The exons are denoted by ▭ the 5'UTR and 3'UTR are marked by ▬ and the introns are denoted by —.

3.3. ubc9 tissue distribution and expression pattern in the developing embryo and gonads

ubc9 mRNA expression was detected in a variety of tissues. The expression of mRNA was highest in the gonads, with a high expression in blood and brain, but low expression in muscle and intestine (Fig. 3). The RT-QPCR results showed that mRNA expression in the embryo and gonads had significant differences. In the developing embryo, *ubc9* transcription was higher in the cleavage stage (CS) than the blastula stage (BS), and the highest level of transcription was observed in the organogenesis stage (OS) (Fig. 4A). In the developmental stages, an extremely low level of expression of *ubc9* was detected at

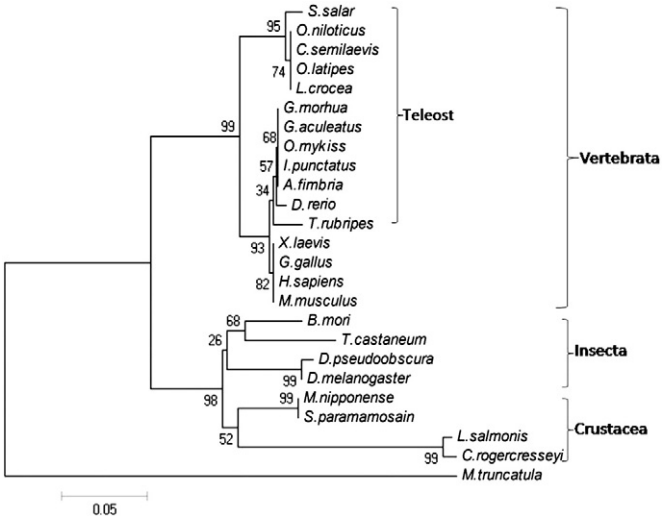

Fig. 2. Dendrogram graphically showing the relations for different organisms based on the amino acid sequence. The relative genetic distances are indicated by the scale bar and the branch lengths. Species names are abbreviated in the tree. *D. rerio* (NP_571426.1), *I. punctatus* (NP_001187932.1), *G. morhua* (ENSGMOP00000017912), *G. aculeatus* (ENSGACP00000011777), *A. fimbria* (ACQ58614.1), *O. mykiss* (ACO08675.1), *T. rubripes* (ENSTRUP00000040080), *L. crocea* (ACI25611), *C. semilaevis* (JQ765845), *O. latipes* (ENSORLP00000016945), *S. salar* (ACI67730.1) *O. niloticus* (XP_003443181.1), *X. laevis* (AAB57736.1), *G. gallus* (BAB68210.1), *H. sapiens* (NP_003336.1), *M. musculus* (NP_001171081.1), *B. mori* (NP_001040407.1), *T. castaneum* (XP_967918.1), *D. pseudoobscura* (XP_001356428.1), *D. melanogaster* (NP_476978), *M. nipponense* (ADD73553.1), *S. paramamosain* (ACJ03792.1), *L. salmonis* (ACO11828.1), *C. rogercresseyi* (ACO11091.1), *M. truncatula* (XP_003611678.1).

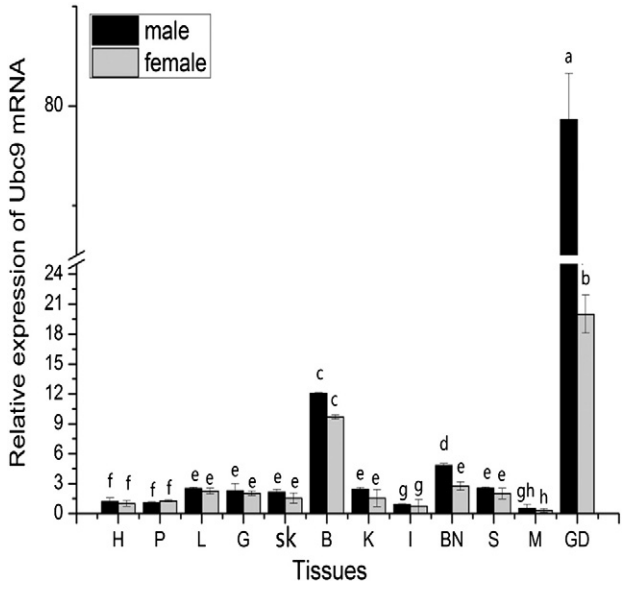

Fig. 3. The expression of *ubc9* in various tissues of *C. semilaevis.* H:heart, L:liver, G:gill, SK: skin, B:blood, K:kidney, I:intestine, BN: brain, S:spleen, M:muscle, P:pituitary, GD: gonad. The data were analyzed by one-way ANOVA followed by Duncan comparison tests using SPSS 17.0. Bars represent triplicate means ± SE from three separate individuals (n = 3). Bars with different letters differed at $p < 0.05$.

the age of 7 days (7D) and 25 days (25D). From 48 day (48D) to 2 years (2Y), the *ubc9* transcription increased in gonad along with age. At age 5 month (5 M) the *ubc9* expression level was sharply increased in the gonads (Fig. 4B) and there were significant differences between the testis and ovary ($p < 0.05$) (Fig. 4B).

3.4. ubc9 expression in the course of sex reversal in tongue sole.

To elucidate whether sex reversal is accompanied by changes in *ubc9* mRNA transcription, *ubc9* mRNA expression was examined by RT-PCR at one year of age after hatching. An extremely high level of *ubc9* mRNA was detected in the gonads of the neo-males produced by temperature treatment, with low expression in the female (Fig. 5).

3.5. Expression, purification of the recombinant proteins

The PET-32-Ubc9 recombinant protein was successfully expressed by inducing the T7 promoter with IPTG. BL21 *E. coli* cells that were transformed with a PET-32a-Ubc9 plasmid and induced with IPTG produced a protein of 35 kDa (Fig. 6: lane 4) which was absent in the non-induced cells (Fig. 6: lane 3). Purification by binding to a Ni Sepharose 6 fast flow column yielded a single band of 35 kDa for Ubc9 (Fig. 6: lane 6). The purified protein showed the same mobility as the induced recombinant proteins from the crude cultures on an SDS-PAGE, and thus confirmed the authenticity of the proteins.

3.6. Expression analysis after protein injection

Injection of the Ubc9 protein into the ovary of *C. semilaevis* resulted in significant change in the expression of FSE and *fst* gene from the injection starting time to 96 h (Fig. 7) ($p < 0.05$). The expression level of *fst* mRNA increased from 12 h to 48 h, with the highest expression level at 48 h after the protein injection, and there was a significant difference between Ubc9 protein injection group and control group ($p < 0.05$). The expression level then decreased from 48 h to 96 h, followed by a recovery to normal level at 96 h (Fig. 7A). However, the expression of FSE gene mRNA first decreased and then increased, and finally returned to normal levels, with the lowest expression of both *cyp19a1a* and *foxl2* at 12 h, and the lowest expression of *ctnnb1* at 48 h. The fact that the expression levels of FSE genes were found to be at first down-regulated and then up-regulated, and then to finally recover to a normal level throughout the injection process, suggests that the Ubc9 protein first repressed the expression of FSE gene and then the expression rebounded to a high level.

4. Discussion

Since *ubc9* is an important gene in the SUMO pathway and helps regulate gene expression (Muller et al., 2001; Johnson, 2004), it has attracted considerable attention. One of the important features of Ubc9 is that it is modified in a number of cellular pathways, including the cell cycle, cell proliferation, DNA repair, protein stabilization and protein sub-cellular localization. It has been reported that Ubc9 mediates sumoylation in order to control the development of ovarian follicles (Kuo et al., 2009). In mammals, Ubc9 exerts an effect on Foxl2 cellular localization, stability and transcriptional activity (Marongiu et al., 2010), and Ubc9 is also reported having an important role in

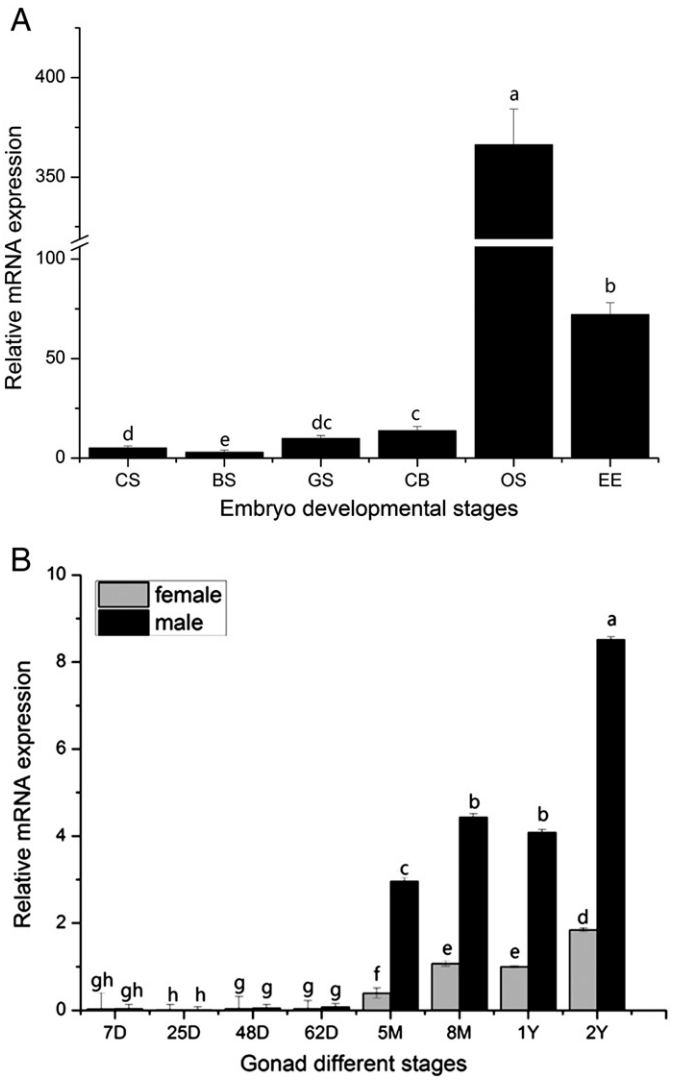

Fig. 4. The expression profile of *ubc9* at different developmental stages of the embryo (A) and the gonads (B) was revealed by real-time quantitative PCR. The *ubc9* mRNA amount was normalized to the β-actin transcript level. The data were analyzed and bars represent the triplicate mean ± SE from three separate individuals (n = 3). Bars with different letters differed with statistical significance ($p < 0.05$). A: CS:cleavage stage, BS: blastula stage, GS: gastrula stage, CB: closure of blastopore stage, OS: organogenesis stage, EE: embryo encircled stage. B: 7D: 7 day age, 25D: 25 day age, 48D: 48 day age, 62D: 62 day age, 5 M: 5 month age, 8 M: 8 month age, 1Y: 1 year age, 2Y: 2 year age.

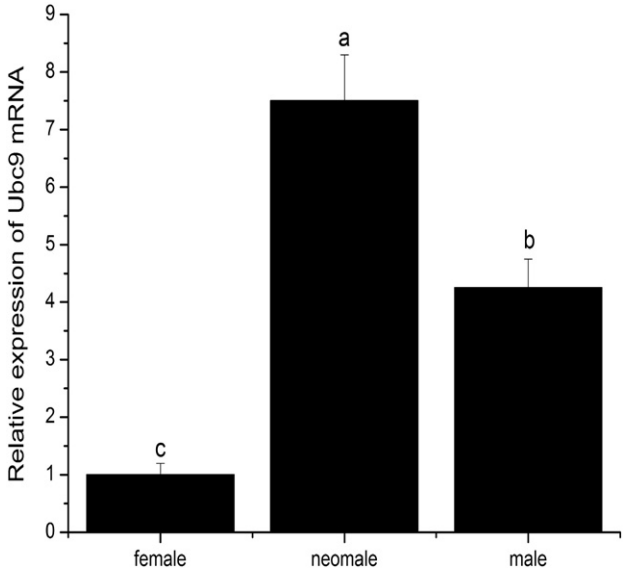

Fig. 5. The expression of *ubc9* among normal ovary, normal testis and temperature treated ovary in one-year old *Cynoglossus semilaevis.* The data were analyzed and bars represent the triplicate mean ± SE from three separate individuals (n = 3). Bars with different letters differed at $p < 0.05$.

the development of oocytes (Ihara et al., 2008). Thus, the cloning and characterization of the *ubc9* gene will provide valuable information for analyzing the function of Ubc9 in both development and sex modification. Many of *ubc9* genes of the teleosts have been cloned and sequenced. To date, *ubc9* gene has been characterized in certain teleosts, such as *D. rerio* (Yuan et al., 2010), *Ictalurus punctatus* (Chen et al., 2010) and *L. crocea* (Zhou et al., 2009a). In the present study, we isolated the full-length cDNA and genomic sequences of *ubc9* genes from *C. semilaevis*. The *ubc9* gene had six exons and five introns in the teleosts and mammals, and the length of each exon was the same as in the vertebrates, such as *T. rubripes* and *Gadus morhua*, but the length of the introns was different. The third intron of *ubc9*, which is 194 bp long, is larger than the one found in *G. morhua* because of the microsatellite repeats: $(TA)_{32}$. The counterpart in *T. rubripes* also displayed microsatellite repeats i.e. $(CA)_3$, $(TG)_3$, $(TC)_3$. The fourth intron of *ubc9* in *T. rubripes* is only 160 bp and in *G. morhua* it is 1133 bp. It is possible that some portion of the fourth intron was lost in evolution, as in the case of *T. rubripes* (Venkatesh and Brenner, 1997). The deduced Ubc9 AA sequence was 76%–100% identical with other species.

Phylogenetic analysis revealed that the gene of *ubc9* was branched into two ways in fishes. One branch had high homology with the *C. semilaevis*, such as *L. crocea, O. latipes* and *O. niloticus,* and another had high homology with mammals. An alignment was carried out among *C. semilaevis, H. sapiens and D. rerio.* The results suggested that in zebra fish, the sequence of Ubc9 AA was 98% degree with human, and 94% degree with *C. semilaevis.* A comparison of the homology among vertebrates indicated that with the base substitution, some of the fishes had a high homology with the mammals, such as *D. rerio, I. punctatus, O. mykiss* and so on.

The ubiquity pathway regulates the G1/S transition (of cell divisions) during mitosis and is necessary for the G2/M transition and progression during the course of organogenesis in vertebrates (DeSalle and Pagano, 2001; Nowak and Hammerschmidt, 2006). In mouse, Ubc9 deficiency in early postimplantation embryos resulted in mitotic anomalies, including chromosome condensation and segregation defects (Artus et al., 2006). In arthropods, Ubc9 is related to embryonic development (Hashiyama et al., 2009). In zebra fish, reduction of Ubc9 activity caused widespread apoptosis during the embryo stage (Nowak and Hammerschmidt, 2006), and the further analysis reveals that cells undergoing p53-dependent apoptosis (Yuan et al., 2010). In *C. semilaevis,* the results of RT-PCR showed that the expression of *ubc9* was detected in the early stages of *C. semilaevis* embryo. It suggested that *ubc9* mRNA was possibly maternally provided in the early stage of the embryo. The lowest expression in the BS, and the expression increased from BS to OS (Fig. 4A). This implied that *ubc9* involved the development of embryo. The highest expression in OS suggested that Ubc9 plays an important role in embryogenesis and organogenesis. In zebra fish and oriental river prawns, the similar expression pattern was observed. (Nowak and Hammerschmidt, 2006; Zhang et al., 2010).

The expression of *ubc9* was analyzed in a variety of tissues (heart, liver, gill, skin, blood, kidney, intestine, brain, spleen, muscle, pituitary gland and gonads), and at different developmental stages of gonads. The results of *ubc9* expression in the different tissues revealed that *ubc9* was ubiquitously distributed. The high expression levels in the gonads suggested that SUMOylation was highly active during mitosis and meiosis. In *L. crocea* and *Eriocheir sinensis,* a similar expression profile was observed (Zhou et al., 2009a; Wang et al., 2012). In *D. melanogaster* and *Coprinus cinereus,* Ubc9 regulated the processes during meiosis and mitosis in the developing testis and ovary (Apionishev et al., 2001; Sakaguchi et al., 2007).

Oogenesis and spermatogenesis are actively driven by mitosis and meiosis with alternating patterns of protein activation (Wang et al., 1996; Grondahl et al., 2000). In mammal, different phases of spermatogenesis required different specialized activities of the Ubc9 (Baarends et al., 2000; La Salle et al., 2008). In the present study, the *ubc9* expression level was analyzed in the developing gonads. At the age of 5 M, both the males and females were immature, but the start of meiosis in the spermary lobule and ovarian cavity shaping was observed (Ma et al., 2006). In addition, from 5 M to 2Y, the gamete was matured gradually in the gonad. The concomitant change in *ubc9* expression with gonadal development and meiosis of the testis and ovary suggested a close relationship between gametogenesis and *ubc9* transcription in half-smooth tongue sole. A similar expression profile was found in *E. sinensis,* transcripts from *ubc9* genes were expressed in the gremlin during oogenesis and spermatogenesisgenes (Wang et al., 2012).

In addition, the expression of *ubc9* differed significantly between testis and ovary ($p < 0.05$). According to Ma et al. (2006), the results showed that the expression of *ubc9* was very low before 62D in the ovary and testis, but the expression level was sharply increased at 5 M. The expression level was constantly increased from 5 M to 2Y,

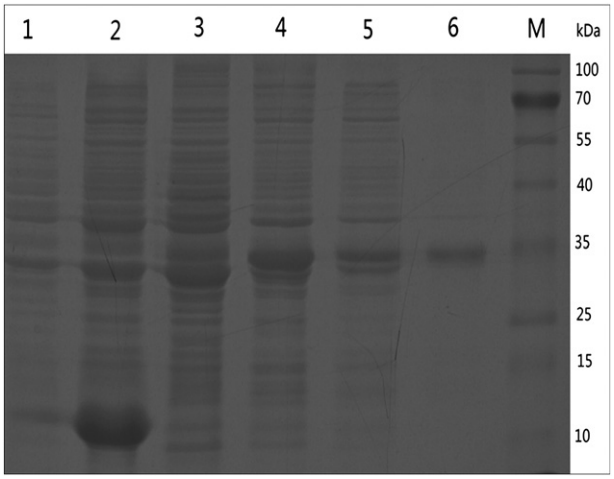

Fig. 6. SDS-PAGE analysis of expressed PET-32a-Ubc9 fusion protein in *E. coli* BL21. M: protein marker; lane 1: un-induced PET-32a; lane 2: induced PET-32a; lane 3: un-induced PET-32a-Ubc9; lane 4: induced PET-32a-Ubc9; lane 5: remaining protein after purification by Ni column; lane 6: Ubc9 protein purified by Ni column.

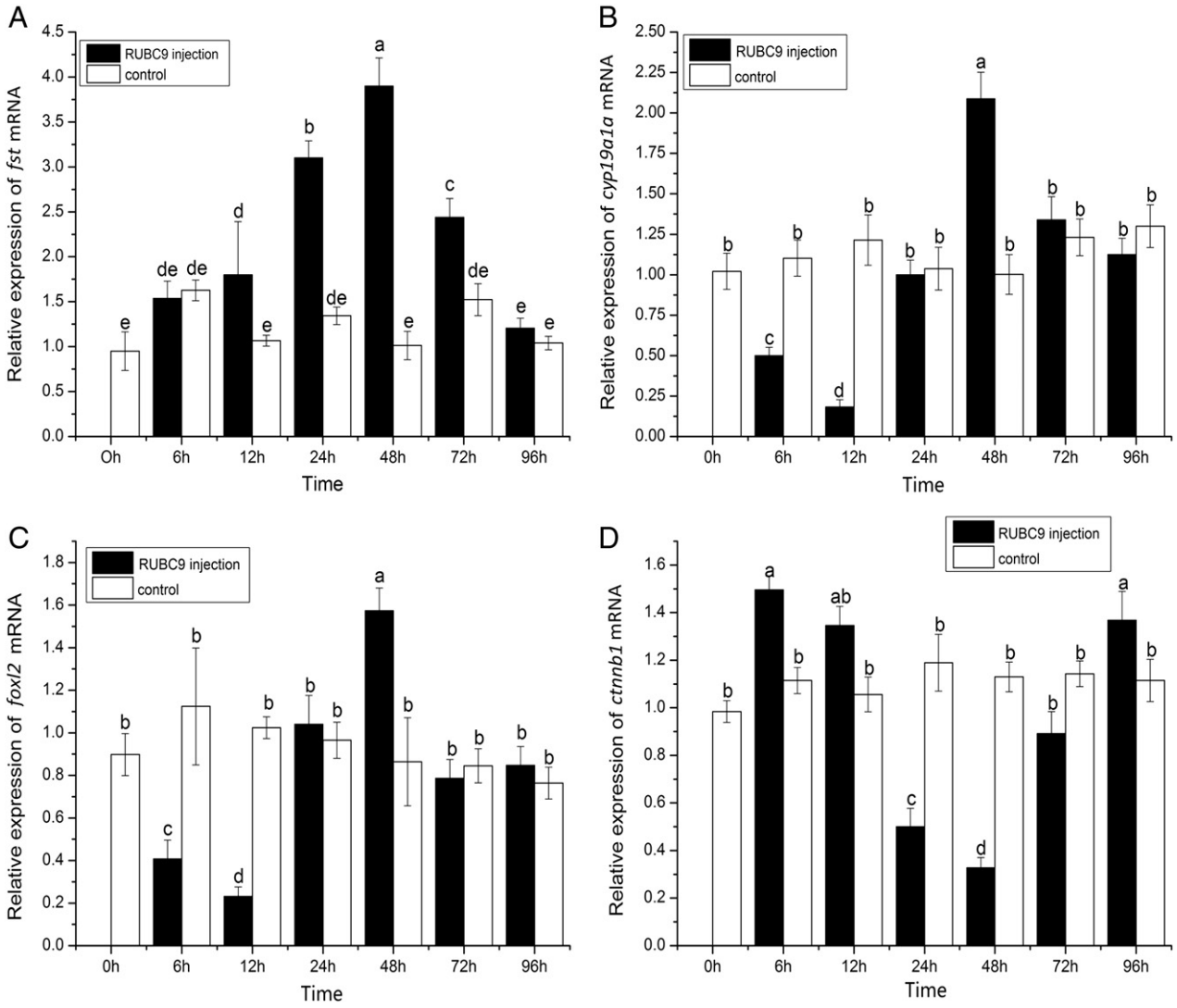

Fig. 7. Expression of *fst* (A), *cyp19a1a* (B), *foxl2* (C) and *ctnnb1*(D) gene after injection Ubc9 protein at 0 h, 6 h, 12 h, 24 h, 48 h, 72 h, 96 h, after injection. Data were analyzed by one-way ANOVA followed by Duncan comparison tests using SPSS 17.0.

with the highest expression level at 2Y, and there was a significant difference between testis and ovary ($p < 0.05$). According to Ma et al. (2006), both the males and females were immature at age of 5 M, but the start of meiosis in the spermary lobule and ovarian cavity shaping was observed, and partial sperm was found at age of 5 M in male, and then more sperm was found at age of 2Y female mature later than male, and someiotic activity was lower in the females at the same stage from 5 M to 2Y. The concomitant change in *ubc9* expression with gonadal development and meiosis of the testis and ovary suggests a close relationship between these events in half-smooth tongue sole. The neo-males were those fish with a genetic female sex but male phenotypic sex (Chen et al., 2008a). A high level of expression was detected in ovaries treated with a high temperature, an intermediate level in normal males and a low level in normal female. These results indicate that Ubc9 plays an important role in sex reversal in these fish.

Recently, it was reported that somatic cells could be induced to become pluripotent stem cells by means of a recombinant protein without the need for a carrier (Zhou et al., 2009b). In this study, the Ubc9 protein was expressed and purified using a Ni column. The expression levels of FSE and *fst* gene were significantly changed after the injection of the Ubc9 protein. Thus, we speculated that protein transduction would perform after injection of recombinant protein of Ubc9 into the ovary. Furthermore, according to Kuo et al. (2009), Ubc9 protein interacted with Foxl2 protein and when Ubc9 protein was increased, the *foxl2* transcription level was decreased. Transcription factors are a main target for sumoylation, a process that usually lead to repression by a poorly understood mechanism (Girdwood et al., 2004; Hay, 2005). Recently, Ihara et al. (2008) reported that Ubc9 is a component of nuclear speckles and regulate transcription via RNA processing. Additionally, Ubc9-mediated sumoylation was involved in Foxl2's activity as a repressor of StAR gene transcription. The *foxl2* transcription was decreased and StAR gene transcription was increased. However, the level of StAR transcripts was up-regulated and closely related to the overall steroidogenic output during the reproductive cycle in male rainbow trout (Kusakabe et al., 2006). According to Hasegawa, the testes of newborn StAR knockout mice exhibited histological features of apoptosis and sperm maturation was delayed; however, the ovaries of StAR knockout mice showed normal features (Hasegawa et al., 2000). The above data suggested that StAR played an important role in testis development, thus the expression of *cyp19a1a, ctnnb1*was repressed and *fst* was enhanced when the StAR transcription was increased. Therefore, it is reasonable to think that injection of Ubc9 protein down-regulated the transcription of the FSE genes and up-regulated *fst* gene expression.

In conclusion, we cloned the full-length (of) cDNA and genomic sequences of *ubc9* in *C. semilaevis*. The *ubc9* expression levels in

various tissues, as well as at different stages of the embryo and gonads were detected by RT-PCT, and the results indicated that Ubc9 plays an important role in both embryogenesis and gametogenesis. Different *ubc9* expression levels in the testis, ovary and the ovary treated with a high temperature were observed. These results implied that Ubc9 plays an important role in sex reversal. Moreover, after injection of the Ubc9 protein, the expression of *fst* was up-regulated and the FSE genes were down-regulated, implying that the Ubc9 protein repressed the FSE gene transcription. However, further study will need to be carried out to elucidate the mechanism.

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