Characterization of *Nanog* Gene Involved in the Gonadal Development in Pearlscale Angelfish (*Centropyge Vrolikii*)

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Abstract

The homeodomain transcription factor *Nanog* plays crucial roles in the embryonic/gonadal development and maintenance of embryonic stem cells (ESCs), interacting with transcription factors such as *Oct4* and *Sox2* in mammals. Nevertheless, knowledge of its exact pathways to molecular mechanism remains weak in teleosts, and unexploited in Pearlscale angelfish *Centropyge vrolikii.* The role of *Nanog* in the gonadal development and sex reversal of *C. vrolikii* is worth focusing. In this study, we isolated and characterized a *Nanog* homolog in *C. vrolikii* named as *Cv-Nanog* to understand the expression pattern of the gonadal development. The full-length cDNA sequence of *Cv-Nanog* was 2,136 bp in length and encodes a homeodomain protein of 436 amino acid residues. The analysis of phylogeny, the gene structure and western blot provided the evidence that *Cv-Nanog* was homologous to *Nanog* gene of mammalian. The protein sequence comparison demonstrated that *Cv-Nanog* shared the high similarity with orthologs of other vertebrates in the conserved homeodomain. The analysis of quantitative real-time PCR (qRT-PCR) and western blot showed that *Cv-Nanog* was maternally expressed detectable merely in gonads, and the expression was significantly high in ovaries than in testis. *In situ* hybridization revealed that the transcripts were located in the cytoplasm and membrane of the oocytes in ovaries, and in the testis, *Cv-Nanog* mRNA expression was weakly expressed in sertoli cells, but highly expressed in germ cells. After overexpression of *Cv-Nanog*, the expression levels of pluripotent factors *Sox2* and *Oct4* increased significantly with 21.5-fold and 12.2-fold, respectively. At the same time, the TGF-beta signaling pathway was activated, and the gonadal cells' growth was promoted, and the expression of ovary-bias genes *Cyp19a* and *Foxl2* was up-regulated, the expression of testis-bias genes *Sox9* and *Dmrt1* was down-regulated, and finally the ovarian development was promoted. These results implied that *Nanog* gene may play a crucial role in the process of gonadal development and sexual reversion in *C. vrolikii*. This study is helpful to further understand the molecular regulatory mechanism of *Nanog*, also provides important clues for the future studies in gonadal development.

Highlights

- The *Cv-Nanog* was significantly expressed in the gonads.
- The expression level of *Cv-Nanog* in the ovaries was significantly higher than that in the testis.
- The functional domain of *Cv-*Nanog was highly conserved among teleosts.
- *In situ* hybridization revealed that the transcript was located in the germ cells in the gonads.
- The TGF-beta signaling pathway was activated, and the gonadal cells' growth was promoted after overexpression *Cv-Nanog* in the gonads.

1. Introduction

Studies on the induced pluripotent stem cells (iPSCs) have become hot research topics in recent years. In the past studies, *Oct4*, *Sox2*, *cMyc* and *Klf4* (OSKM) had successfully induced the reprogramming of human somatic cells into iPSCs (Takahashi and Yamanaka, 2006). Subsequently, OSKM had been

regarded as the key factors to obtain iPSCs (Maherali and Hochedlinger, 2008, Zhao et al., 2008), and *Oct4* was considered to be the only indispensable factor for iPSCs induction (Nakagawa et al., 2008). As one of the key genes to maintain the pluripotency and self-renewal ability of the embryonic stem cells (ESCs), *Nanog* gene was found for the first time during the construction of mouse ESCs cDNA library (Wang et al., 2003) and it is specifically expressed in pluripotent tissues such as undifferentiated cells of cell clusters in blastocyst stage (Mitsui et al., 2003). Subsequently, some investigations reported that *Nanog* gene may play an important role in maintaining the pluripotency of ESCs, and can make the stem cells have the ability of self-renewal for a long time independent of LIF/STAT3 pathway (Chambers et al., 2003, Mitsui et al., 2003). *Nanog* had always been considered as a marker gene of stem cells (SCs) in mammals, which can make the SCs tend to differentiate without *Nanog* gene even in the presence of leukemia inhibitory factor (LIF), what's more, the differentiation of ESCs can be promoted after knockdown *Nanog* (Zaehres et al., 2005). Conversely, the self-renewal ability and the expression of pluripotent factors of the ESCs was maintained /reinforced and activated, respectively, after overexpression *Nanog* gene (Mitsui et al., 2003, Darr et al., 2006, Silva et al., 2006).

Nanog gene of mouse encoded 305 amino acids, Nanog protein consisted of a relatively conserved homeodomain (HD), the HOX domain, an amino terminal rich in serine and threonine, and a segment base terminal containing a manifest tryptophan rich domain (WR) (Chang et al., 2009, Das et al., 2011, Pan and Pei 2005, Mitsui et al., 2003). Among the basic structural and functional domains of its protein, the HOX domain played a central role in binding with DNA and interacting with proteins and WR region, the principal functional region of Nanog protein, which can interact with other pluripotent factors to maintain the self-renewal ability of ESCs in mouse (Mullin et al., 2008, Wang et al., 2008). Different from mammals, there wasn't WR domain in the carboxyl terminal of Nanog protein of lower vertebrates such as zebrafish (*Danio rerio*) and salamander (*Salamandra salamandra*) but its HD and amino terminal domain can form a dimer structure to directly regulate the pluripotency of ESCs (Dixon et al., 2010, Schuff et al., 2012).

Due to the important role of *Nanog* gene in the pluripotency maintenance of ESCs or germ stem cell (GSCs), the expression regulation of *Nanog* was researched to reveal the regulatory effects of a variety of transcription factors. There were relatively conservative *Oct4*, *Sox2*, *Klf4* and *Pbxl* binding sites in the proximal promoter region of mammalian *Nanog* which can play an important role in the up regulation of *Nanog* gene expression (Kuroda et al., 2005, Rodda et al., 2005, Chan et al., 2009). As one of the key transcription factors maintaining the self-renewal of ESCs, *Nanog* also plays an important role in iPSCs (Okita et al 2007, Rais et al 2013). The combination of *Oct4*, *Sox2*, *Nanog* and *Lin28* transcription factors can also successfully reprogram human somatic cells return to pluripotent state and have some characteristics of ESCs (Yu et al., 2007). It is worth notice that short-term expression of two components, *Nanog* and *Klf4*, or *Nanog* and *Bmil*, was sufficient to activate the pluripotent regulatory network and reset the pluripotent state in mammals stem cells, and *Nanog* gene can replace *Oct4* or other genes to participate in cell reprogramming in mammals (Moon et al., 2013, Takashima et al., 2014). Whether *Nanog* gene has the similar effect in the regulation of pluripotency in fish has not been reported.

Nanog was highly expressed in undifferentiated ESCs (Chambers et al., 2003), and was also primarily expressed in GSCs (Yamaguchi et al., 2005), fetal testis (Kerr et al., 2008), seminoma and breast cancer (Ezeh et al., 2005) and some adult tissues (Hart et al., 2004). The direct homologues of mammalian *Nanog* gene of fish was identified from the model fish medaka (*Oryzias latipes*) for the first time (Camp et al., 2009), then from the teleost fish, such as zebrafish (Schuff et al., 2012), goldfish (*Carassius auratus*) (Marandel et al., 2012), blunt snout bream (*Megalobrama amblycephala*) (Yu et al., 2017), and so on. Unlike mammals, *Nanog* gene of fish showed a maternal expression pattern which can be detected from unfertilized eggs and had high expression level until blastocyst stage. Additionally, in the early embryonic development, *Nanog* was also obviously expressed in the adult gonads of teleost fish (Wang et al., 2011). And the expression of *Nanog* gene was also detected in some adult tissues of fish, such as the liver of blunt snout bream (Yu et al., 2017), suggesting that it may be relevant to the existence of adult stem cells in this tissue. The role of *Nanog* gene in gonadal development of teleost fish is worthy to further study and exploration.

Pearlscale angelfish (*Centropyge vrolikii*), commonly known as the black tail angelfish, mainly distributed in the Indian Ocean and the Pacific Ocean inhabiting near the coral reefs, is a unique marine ornamental fish with tremendous market potential in China (Dibattista et al., 2016, Fernandez-Silva et al., 2018). At present, *C. vrolikii* is obtained mainly by wild fishing, but due to the increase of market demand and the destruction of ecological environment, the natural yield of *C. vrolikii* decreases year by year, and the artificial breeding is inevitable. However, as a kind of protogynous hermaphrodite and natural sexual reversion fish, the disadvantages of long breeding cycle and difficult artificial breeding become the main restricting factor for the breeding of *C. vrolikii* (Zhong et al., 2021). Therefore, it is indispensable to study the molecular regulation mechanism of gonadal development in *C. vrolikii*. In our previous research, the differential expression genes (DEGs) in gonads at different stages in *C. vrolikii* had been screened using RNA-Seq technology (Zhong et al., 2021). About *Nanog* gene of *C. vrolikii*, which we have designated as *Cv-Nanog*, very little research has been done at the molecular level. In mammals, the regulatory network of *Nanog* had been deeply studied, but the molecular mechanism of the regulatory network in *C. vrolikii* has not been reported. The role in the process of gonadal development is also noteworthy. The present study was aimed at identifying the *Cv-Nanog* which will be cloned and characterized to understand the regulatory network of *Cv-Nanog*. Our findings will provide a new perspective on gonadal development of small sea angelfish, which may have important significance for artificial breeding of *C. vrolikii*.

2. Materials And Methods

2.1 Samples collection and preparation of tissue sections

The fish of *C. vrolikii* were obtained from the Aquatic Product Experiment Center of Fisheries College Jimei University (Xiamen, China). According to our previous research (Zhong et al., 2021), the gonads from four developmental stages were collected, namely ovary at the perinucleolus stage ($O_{□}$), ovotestis at the hermaphroditic stage (O_T), ovary at the yolk vesicle stage ($O_{□}$), and pure testis (T). One part of gonads (0.5 cm^3) were quickly fixed in 4% paraformaldehyde (PFA) at 4℃ for 24 h for further use, others of

gonads and other tissues (kidney, liver, spleen, brain, gill, eye, intestine, and heart) were collected in RNAlater (Ambion) and stored at 4℃ for 24 h, and then transferred to - 80℃ for storage until RNA extraction and qRT-PCR.

The gonads (in PFA) were dehydrated, decolorized, waxed impergnation, and sectioned continuously into 6 μm. Thereafter the sections were stained with hematoxylin-Eosin (H.E), sealed with neutral gum and observed by the microscope.

2.2 Molecular cloning and analysis of *Cv-Nanog*

The primers (P-*Nanog*-F / -R, Table S1 in Supplementary) were designed with Primer 5.0 software based on the gonad transcriptome data by RNA-Seq of *C. vrolikii*. The partial fragment of *Cv-Nanog* was cloned by polymerase chain reaction (PCR) using the cDNAs of ovaries as the templates for amplification. The primers of *Cv-Nanog* for nested PCR were designed with Primer 5.0 to clone the 5' and 3' regions (Table S1 in Supplementary). Referring to the instructions, the total RNA of each tissue was extracted and the first cDNA strand is synthesized from 3.0 μg total RNA samples. The cDNA was diluted 10 times with DEPC water as PCR template. PCR amplification procedure was as follows: 94℃ for 4 min, 35 cycles of 94℃ for 30 s, 51℃ for 30 s, and 72℃ for 1 min, followed by 72℃ for 10 min. The PCR products were separated by agarose gel electrophoresis, and then recovered by gel Recovery Kit (Promega). The target fragments are connected to 19-T vector by DNA connection kit (TAKARA), and then transformed into DH5*α* competent cells. The recombinant product was coated on solid medium adding ampicillin and cultured at 37 ℃ for 16 h, Then the monoclonal cell was selected and detected with M13 universal primer, the positive clones were expanded cultured and sequenced to ensure the accuracy of amplification.

Nucleotide and protein sequences of *Cv-Nanog* were performed and analyzed by National Centre for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The theoretical amino acid composition, PI and Mm were computed by Expasy ProtParam Tool (http://web.expasy.org/protparam/). N-glycosylation sites (N-X-S/T) were predicted with NetNGlyc1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and phosphorylation sites with NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/). Protein domains were predicted by SMART (http://smart.embl.de/). Multiple sequence alignments were compared by BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Interacting pathways of Nanog protein with other proteins were depicted using a STRING database (https://string-db.org/cgi/input.pl). The phylogenetic tree was constructed by using the bootstrap neighbor-joining (NJ) method of MEGA 7.0 (http://www.megasoftware.net) and the bootstrap values were replicated 1000 times.

2.3 qRT-PCR

The gene-specific primers for qRT-PCR were designed by Primer 3.0 software based on the obtained *Cv-Nanog* sequence. The primers of reference genes referred to our previously published peer-reviewed article with *Rps29* as a reference gene for the tissue expression (Zhong et al., 2021). The reverse

transcription products of each sample for qRT-PCR were properly diluted as the templates, and each sample was assayed with three technical replicates. SYBR Green Master Mix (Vazyme) was used for qRT-PCR analysis. The relative fold change of the gene expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. All of the primers were shown in Table S1 in Supplementary.

2.4 In situ hybridization

In accordance with method in our previous experimental (Jiang et al., 2018), the sense and anti-sense probes of *Cv-Nanog* were prepared *in vitro* transcription with the digoxigenin (DIG)-labeled and T7 RNA polymerase before *In situ* hybridization (ISH). Then store at - 80℃ for use. Firstly, the sections were dewaxed with xylene, rehydrated with ethanol (95%, 90%, 85%, 75%, 50%) and cleaned with PBS, and incubated with proteinase K at 37℃ for 30min. Secondly, the sections were carefully placed in a dark box and soaked with the prehybridization solution (Hyb-, no DIG-labeled RNA probe), incubated at 60℃ for 3 h. Thirdly, all the sections were soaked with the hybridization solution (Hyb+, DIG-labeled RNA probe 1.0 ng/mL) and incubated at 60℃ for 16 h. Then the sections washed with Hyb- gradient and PBS and incubated anti-DIG-AP anti-body overnight at 4℃. Finally, the sections washed with PBS and TMNT and stained with BCIP/NBT in the dark. Then, the sections were washed with PBS and sealed with water-soluble sealing agent, and observed under microscope.

2.5 Western blot

The samples (the gonadal tissue at different stages) stored - 80℃ were cut into pieces and homogenized in the tissue homogenizer with the lysis buffer (1 mL per 100 mg) on ice until there is no visible solid. The supernatant is transfered into another precooled clean centrifuge tube and stored at - 80℃ after the homogenate was centrifuged at 10,000 g at 4 ℃ for 10 min.

For SDS-PAGE, firstly, the mixture (protein samples and 5 × SDS-PAGE loading buffer, 4:1) was boiled for protein denaturation at 100℃ for 15min. Then the mixture and prestained protein marker were added to the wells of the 10% separation gel, respectively. After electrophoresis at 80 V for 30 min, the voltage was adjusted to 120 V for about 50 min until the indicator moves to the bottom of the gel and runs out. Secondly, the proteins were transferred from gel to a PVDF membrane with a membrane transferring apparatus under 200 mA for 90 min. The PVDF membrane containing proteins after transfer was washed with PBS or PBST three times for 5 min. Then it was placed in a small box with 5% BSA blocking solution (diluted with 1 × PBS) and incubated at 37℃ for 1 h. Thirdly, the membrane was washed with PBS or PBST three times for 5 min and placed in a hybridization bag and soaked by adding *Nanog* and *β-actin* primary antibodies at 1:500 and 1:200 dilutions, respectively. The bag was then sealed and incubated at 4℃ overnight. On the second day, the membrane was washed with PBS three times for 10 min and incubated with diluted (1:10000) HRP-conjugated secondary antibody (goat anti-rabbit-HRP) at room temperature for 1 h. Finally, the PVDF membrane was washed with PBS three times for 10 min and faced up by adding 2 mL of chromogenic agent until significant brown coloration bands could be found, and observed under e-Blue touch imager.

2.6 Liposome transfection and cell culture

The fish washed and soaked in a bucket containing 1‰ commercial hypochlorous acid disinfectant for 1 h, and then add 3-4 drops of eugenol (about 0.1‰ of water volume) for anesthesia. Then the gonads were dissected carefully and washed three times in PBS containing amphotericin B and double-antibody. The tissues were chopped (about 1 mm^3), washed three times with PBS. The tissues were seeded evenly in a six-wells cell culture plate, infiltrated with 1 mL cell culture medium (L-15 adding 15% FBS, EGF, HGF, β-FGF, CMC, N-AG and 2-Me) and cultured at 27°C for two days. On the third day, 2 mL cell culture medium was added for further culture.

According to method in our previous experimental (Xu et al., 2021), the CDS without the stop codon of *Nanog* gene was inserted into the pEGFP-N1 vector to obtain the overexpression vector (pNanog-N1), the double digestion primers were designed through website (https://crm.vazyme.com/cetool/singlefragment.html) (primers: O-Nanog-F2 / -R2). The purified plasmid was extracted by Endo-Free Plasmid DNA Maxi Kit (E.Z.N.A.® Omega) for the liposome transfection.

On the day of transfection, the medium was changed to Opt I MEM 5 mL. Refer to the user manual of Lipofectamine™ 2000, the solution A and solution B were prepared in a new 1.5 ml EP tube. The dosage of each well in a 6-well plate is as follows: solution A: 2 μg pNanog-N1 plasmid was added to 250 μL Opt I MEM medium standing at room temperature for 5 min, solution B: 10 μL Lipo2000 was added to 250 μL Opt I MEM medium standing at room temperature for 5 min. The solution A and B were mixed staying at room temperature for 20 min. Then the mixture was added to the 6-well plate, respectively, and shaked gently to make them evenly contact the cells, and cultured in CO_2 incubator. After contacting the liposome-DNA complex with cells (tissues) for 6 h, it was replaced with complete medium containing 15% FBS for culturing. The cells were observed at 24 h and 48 h after transfection, and the cell growth was recorded. The RNA of the gonads (cells) after transfecting was extracted and reverse transcribed them into cDNA for the detection of the pluripotency factor and the sex-related genes were detected by qPT-PCR. All of the primers were shown in Table S1 in Supplementary.

2.7 Statistical analysis

All statistical analysises were performed using SPSS20.0 software, and the relative expression levels were compared by One-way ANOVA. The level of significance was less than 0.05.

3 Results

3.1 Cloning and characterization of *Cv-Nanog*

The 3’ untranslated region (3’ UTR) and 5’ UTR were obtained by 3’ and 5’ RACE PCR. The full-length cDNA of *Cv-Nanog* was spliced by bl2seq and confirmed by head to toe PCR. The nucleotide and deduced amino acid sequence were shown in Fig. S1 in Supplementary. The full-length cDNA of *Cv-Nanog* was 2,136 bp composed of a 142 bp 5’-UTR, a 683 bp 3’-UTR and an ORF (nt 143 to 1,453) which encodes a

436-amino-acid protein (*Cv*-Nanog). *Cv*-Nanog was located in the nucleus (k-NN Prediction, k = 23), with Ser (11.0%) and Ala (10.8%) as the highest content of amino acid composition. The estimated molecular weight (MW) was 47.50 kDa, with a theoretical isoelectric point (pI) of 7.29 and grand average of hydropathicity of -0.856. There are 104 phosphorylation sites (Ser: 48, Thr: 34, Tyr: 22), 6 glycosylation sites and no N-terminal signal peptide cleavage site or transmembrane helix in *Cv*-Nanog (Table S2 in Supplementary). *Cv*-Nanog contained one conserved DNA-binding domain: HOX domain (aa: 222-284) (see Fig. S1 in Supplementary). The 3'-UTR contained a putative polyadenylation signal (AATAAA) and 20 nucleotides upstream of the poly (A) tail (see Fig. S1 in Supplementary).

3.2 Phylogenetic analysis of *Cv*-Nanog

Multiple sequence alignment of Nanog for homology analysis was performed between *C. vrolikii* and other known teleosts. The Nanog proteins have highly variable N-terminal and C-terminal domains and highly conserved homologous domains among species. *Cv*-Nanog showed the high identity of 47.21% - 84.47% in overall protein sequence with that from teleosts: *Morone saxatilis* (84.47%), *Sparus aurata* (80.09%), *Perca flavescens* (72.95%), *Perca fluviatilis* (72.95%), *Labrus bergylta* (72.94%), *Notolabrus celidotus* (72.73%), and *Acanthochromis polyacanthus* (58.26%) respectively (see Fig. S2 in Supplementary, Table 1). All of these HDs have a conservative motif, YKQVKTWFQN. The results of multiple sequence alignment also showed that the functional domains (HOX) of Nanog were highly conserved among teleosts with the sequence identity of 73.16% - 92.06% which was consistent with the HOX domain structure of the mouse (*Mus musculus*) with the sequence identity of 47.5% of the mouse (*Mus musculus*) HOX domain (Table 1).

The phylogenetic tree elucidated the evolutional relationship of Nanog in teleosts (see Fig. S3 in Supplementary). The teleosts group consisted of three subgroups: Salmoniformes, Gadiformes and Perciformes. The *Cv*-Nanog belonged to the Perciformes subgroup, including *A. polyacanthus*, *M. saxatilis*, *P. flavescens*, *P. fluviatilis*, *Labrus bergylta*, *N. celidotus* and *A. polyacanthus*. The phylogenetic tree elucidated that the *Cv*-Nanog was the closest relationship to *M. saxatilis* in the Perciformes sub-clade.

3.3 Protein-protein interactions for Nanog

The scores of neighborhood, gene fusion, co-occurrence, co-expression and homology scores with the interacting proteins were considered. Nanog was a main member of the NOS triad responsible for stem cell pluripotency and maintenance. Protein-protein interacting networks of Nanog along with other factors (Table S3 in Supplementary, see Fig. S4 in Supplementary) involving pluripotency was investigated using STRING. It revealed that *Cv*-Nanog interacted with the other pluripotency marker proteins such as Oct4 (POU domain, class 5, TF1), Sox2 (Transcription factor Sox-2), Klf4 (Kruppel-like factor 4), Lin28a (Protein lin-28 homolog A), cMyc (Transcription factor that binds DNA in a non-specific manner) and so on (Table S3 in Supplementary).

3.4 Expression of *Cv-Nanog*

The expression of *Cv-Nanog* in various tissues of *C. vrolikii* was analyzed by semi-RT-PCR (Fig. 1 A) and qRT-PCR (Fig. 1 B). The results showed that *Cv-Nanog* was expressed strongly in ovary. Moreover, the highest expression level occurred in ovary at the yolk vesicle stage ($O_{\square}$) ($p < 0.01$) which was higher (about 8.9 fold) than in ovary at the perinucleolus stage ($O_{\square}$), was much higher (about 19.3 fold, 20.9 fold) than in the gonads at hermaphroditic stage (O_T) and testes, respectively. The expression of *Cv-Nanog* was expressed lowly in the rest of the tissues (Fig. 1).

The expression of *Cv*-Nanog protein in gonads was detected by western blot. Anti-Nanog antibody detected a band of about 37 kDa in the protein of gonads at each stage, which was similar to the predicted size of *Cv*-Nanog (37 kDa), indicating that *Cv*-Nanog was expressed in gonads at each stage. Among them, *Cv*-Nanog was most strongly expressed at means of $O_{\square}$, then gradually decreased in the $O_{\square}$, O_T and T (Fig. 1 C).

3.5 Distribution of *Cv-Nanog*

In situ hybridization on 5-μm-thick paraffin sections were performed to identify the distribution pattern of *Cv-Nanog* in different developmental gonads. The ovary of $O_{\square}$ stage was dominated by the oocytes at stage □ with small cell volume and no yolks (Fig. 2 A-1), while the ovary of $O_{\square}$ stage was dominated by oocytes at stage □ with large volume and rich yolks (Fig. 2 B-1). The gonads were coexistence of male and female germ cells at O_T stage (Fig. 2 C-1). By ISH, *Cv-Nanog* mRNA expression was mainly concentrated on the cytoplasm and membrane of the oocytes. At $O_{\square}$ stage, the positive signal was mainly detected in the cytoplasm of the oocytes at stage □ (Fig. 2 A-3). At the $O_{\square}$ stage, the positive signals were also detected on the membrane of the oocytes at stage □ (Fig. 2 B-3). At O_T stage, *Cv-Nanog* mRNA expression was higher in oocytes than sperm (Fig. 2 C-3). In the testis, there were a number of seminiferous lobule and each lobule contains different developmental stages of male germ cells (Fig. 2 D-1). The results showed that *Cv-Nanog* mRNA expression was weakly expressed in sertoli cells, but highly expressed in sperm (Fig. 2 D-3).

3.6 Expression of sex-related genes after *Nanog* overexpression

During the two days of gonadal tissue culture, a few cells migrated from around the tissue. After overexpression of *Cv-Nanog* (24 h), the growth of cells was significantly faster than that of normal cells, and the cell morphology became fuller and more tension (Fig. 3). The qRT-PCR results showed that the expression levels of pluripotent factors, *Sox2*, *Oct4*, *Klf4* and *cMyc* increased significantly after overexpression of *Cv-Nanog*, of which *Oct4* and *Sox2* increased most significantly, 21.5-fold and 12.2-fold, respectively (Fig. 4 A). In addition, the expression of sex-related genes also changed in varying degrees. The expression of ovary-related gene *Cyp19a* increased significantly, and the expression of *Foxl2* and *sf-1* also increased significantly (Fig. 4 B). The expression of testis-related genes *Dmrt1* and *Sox9* decreased significantly (Fig. 4 B). In the regulatory network presumed of gonadal development mediated by *Cv-Nanog*, Nanog interacts with Sox2 and Oct4 to activate the self-renewal ability of cells, and then activate the TGF-beta signaling pathway and promote the gonadal growth (Fig. 5).

4. Discussion

Since the discovery and isolation of the pluripotent gene *Nanog* in mouse ESCs for the first time (Wang et al., 2003), its role in embryonic development and maintaining pluripotency in ESCs and the regulatory networks had been deeply studied (Chambers et al., 2003, Darr et al., 2006, Mitsui et al., 2003). From then on, *Nanog* had also been reported in birds (Canon et al., 2006, Lavial et al., 2007) and some model organisms, medaka (Camp et al., 2009, Wang et al., 2011) and zebrafish (Schuff et al., 2012, Theunissen et al., 2011). Among the non model fish, there were a few studies on *Nanog* in goldfish (Marandel et al., 2012), *Paralichthys olivaceus* (Gao et al., 2013) and blunt snout bream (Yu et al., 2017). However, little was known about *Nanog* gene of small sea angelfish. In this study, we cloned *Nanog* of *C. vrolikii*, an important marine ornamental fish, and analyzed its sequence characteristics and expression pattern, defined *Cv-Nanog* as the *Nanog* ortholog because of its conserved features that we hereby described, so as to lay a foundation for further study on the mechanism of cell differentiation and gonadal development of *C. vrolikii*.

In this study, a total of 2,136 bp cDNA fragment of *Cv-Nanog* was obtained with encoding 436 amino acids. Like medaka (Wang et al., 2011), zebrafish (Theunissen et al., 2011) and blunt snout bream (Yu et al., 2017), *Cv*-Nanog contains a conserved HOX domain, located at 222-284 (Fig. 2). In addition, the HOX region also contained a conserved YKQVKTWFQN motif that was speculated to be a nuclear localization signal, similar to that observed in human Nanog (Chang et al., 2009, Do et al., 2007, Pan and Pei, 2003). The C-terminal of Nanog in mammalian contained a tryptophan pentapeptide repetitive sequence (Chambers et al., 2003, Hart et al., 2004, Medvedev et al., 2009), some studies also pointed out that the WR region played an important role in the interaction with other pluripotent network regulatory proteins (Mullin et al., 2008, Wang et al., 2008), but a similar repetition of W was not found in Nanog of teleost fish and birds. Structurally, they may not have the conserved function as Nanog of mammalian. However, later studies confirmed that Nanog can play an effective role in the process of cell reprogramming in mammals, birds and teleost fish (Theunissen et al., 2011), indicating that it had a conserved function in vertebrates. As an important component structure of *Nanog*, HOX domain can recognize and bind specific DNA sequences to play its role (Jauch et al., 2008). Multiple sequence alignment found that except the conserved Hox domain, the conservation of other sequences was relatively low. The sequence identity of full-length *Cv*-Nanog protein was lower (16.5%) compared to that of the mouse Nanog (Table 1). In zebrafish, the sequence identity of *Dr*-Nanog was much lower (13%) compared to that in mouse whereas they were functionally conserved (Theunissen et al., 2011). It was speculated that *Nanog* gene had the characteristics of rapid evolution and rich diversity. The phylogenetic tree elucidated that the *Cv*-Nanog belonged to the Perciformes subgroup with the closest relationship to *M. saxatilis* in the Perciformes sub-clade. These showed that the HOX domain of Nanog was very conservative in evolution and very important in the function.

Gonadal development is crucial to the growth and reproduction of fish. An army of studies had demonstrated that *Nanog* gene played essential roles in embryogenesis, gonadal development and maintaining the pluripotency and self-renewal ability of ESCs in vertebrates (Ben-Porath et al., 2008, Clark

et al., 2004, Silva et al., 2006, Wang et al., 2011). The expression of *Cv-Nanog* in tissues showed that in adult tissues, the transcript of *Cv-Nanog* was specifically expressed in gonads at different developmental stages with much higher in ovaries than that in testis, and the expression of *Cv-Nanog* was relatively low (or even almost not) in other adult tissues (Fig. 5). The expression of *Nanog* in the gonads and early developmental embryos was reported, too (Ambady et al., 2010, Ben-Porath et al., 2008, Beltrami et al., 2007, Chambers et al., 2007). In humans, *Nanog* was mainly expressed in ESCs, and also in testes and ovaries (Clark et al., 2004), and ovarian cancer cells (Hoei-Hansen et al., 2005, 2007). In mouse, the expression of *Nanog* was significantly higher than that in heart, kidney, spleen, liver, and differentiated cells (Hart et al., 2004, Ventea et al., 2012). In medaka, the expression of *Nanog* was exhibited in the gonads, predominantly in pluripotent cells (Camp et al., 2009, Wang et al., 2011). *C. vrolikii* is a naturally reversed fish, the mark of the beginning of the gonad reversal is the male germ cells beginning to appear in the ovary,named hermaphrodite, and then the male germ cells are further differentiated, finally the gonad was transformed into pure testis (Zhong et al., 2021). In this study, there were differences in the expression of *Cv-Nanog* in gonadal tissues at different developmental stages with the level $O_{\square}$ > $O_{\square}$ > O_T > T in turn (Fig. 5). It is speculated that *Nanog* were likely to be indispensable in the process of the gonadal development, which may also be closely related to the maternal expression pattern of *Nanog* (Schuff et al., 2012, Wang et al., 2011, Yu et al., 2017).

In the present study, we indicate that in fish, *Cv-Nanog* was expressed in both male and female gonads, further confirming that *Cv-Nanog* was the functional homolog of mammalian *Nanog*. However, it was not clear whether *Nanog* actually exerts a biological function in gamete maturation. In ovaries, *Cv-Nanog* RNA expression was localized in the cytoplasm of the oocytes at stage □ (Fig. 2 A1-A3), and it was only localized in the membrane of the oocytes with no positive signal in yolk granules at stage □ (Fig. 2 B1-B3). In O_T stage, the *Nanog* RNA expression was distributed in both oocytes and sperm (Fig.6 C1-C3). On the other hand, in testis, *Cv-Nanog* RNA was highly expressed in the nucleus of cells in the periphery where spermatogonia was located and weakly expressed in sertoli cells (Fig. 2 D1-D3). The expression of *Cv-Nanog* in the gonadal germ cells was comparable to mouse and chicken (Canon et al., 2006, Lavial et al., 2011, Mitsui et al., 2003, Yamaguchi et al., 2005). Inconsistently, the *Nanog* RNA expression was present in spermatogonia or oogonia in medaka (Wang et al., 2011), in oogonia and early stage of oocytes in ovary, or in spermatogonia, spermatocytes, and spermatids in testis in blunt-snout bream (Yu et al., 2017). According to the current study of western blot, *Cv*-Nanog protein was most strongly expressed in $O_{\square}$, then gradually decreased in $O_{\square}$, O_T and T (Fig. 5 C). The results of western blot analysis were compatible with those of qRT-PCR, suggesting that *Nanog* gene was expressed in gonads with highly in ovaries. Thus, the potentiating presence of *Cv-Nanog* mRNA and protein in ovary could be argued as its maternal inheritance, which was also agreement with the observations in teleosts (Camp et al., 2009, Gao et al., 2013, Marandel et al., 2012).

The studies demonstrated that the *Nanog* overexpression was indeed involved in regulating pluripotency reprogramming in mouse ESCs (Silva et al., 2006) and it can increase the proliferation rate and regulate the cell cycle in human ESCs (Zhang et al., 2009). The transient overexpression *Nanog* could activate the

expression of *Oct4* (5-fold), *cMyc* (2-fold), and *Sall4* (5-fold) in somatic cells in porcine fetal fibroblast (PFF) (Zhang et al., 2011) and it could significantly increase the expression *Oct4*, *cMyc* and other genes in the gonads cell lines (LYCO and LYCT) of large yellow croaker (*Larimichthys crocea*) (Xu et al., 2021). Thereby, the overexpression of *Nanog* can recover the pluripotency of cells to improve the self-renewal ability of cells. Similarly, in this study, the growth rate of cells after *Nanog* overexpression was significantly accelerated (Fig. 3), this may be related to the activation of *Oct4*, *Sox2*, *Klf4*, and other pluripotency factors (Fig. 4), which can recover the pluripotency of cells to improve the self-renewal ability of cells. What's more, the expression of sex-related genes of *C. vrolikii* was also different after *Nanog* overexpression (Fig. 4). In the core transcriptional network (Fig. 5), *Cv-Nanog* interacts with *Sox2* and *Oct4* to activate the self-renewal ability of cells, which will activate the TGF-beta signaling pathway and promote the gonadal development. Then, the Follicle-Stimulating Hormone (FSH) was promoted, the cAMP increased, and the expression of *Cyp19a* and *Foxl2* was up-regulated the expression of *Sox9* and *Dmrt1* was down-regulated and eventually the ovarian development was promoted. The heightened abundance of the sex-related genes in the gonads suggested its participation in maternal inheritance and the gonadal development. Some studies had represented that *Lr-Nanog* may be involved in the development of undifferentiated germ cells in *Labeo rohita* (Patra et al., 2018). It would be of interest to clarify this particular aspect in the future of *Nanog*, it would also be fascinating to carry out the studies of *Nanog* in the regulatory mechanisms and pathways involved in gonadal development and maintenance of germ cells.

5. Conclusion

In summary, this study provided the full-length cDNA sequence of *Nanog* gene in *C. vrolikii*. By the gene structure, protein alignment, phylogenetic tree, expression pattern, and western blot analysis, we deduced that *Cv-Nanog* was the ortholog of *Nanog* of mammalian and teleost. The relatively conserved HOX domain of *Nanog* of teleosts indicated that it might share some common biological functions with mammalian counterparts, particularly in stem cell maintenance and gonadal development. The results of qRT-PCR and western blot in tissues demonstrated that *Cv-Nanog* was maternally inherited, expressed during the gonadal development, particularly high in ovaries. ISH revealed that *Cv-Nanog* RNA exhibited a sustained expression pattern in ovaries but specific in spermatogonia in testis. *Nanog* overexpression experiment in cell showed that *Cv-Nanog* may have a conservative function of regulating pluripotency in *C. vrolikii*, and it may be a key factor in promoting gonadal development. The results will lay a foundation for the next study of gene function, gonadal development and the exploration of fish iPSCs.

Declarations

Ethics approval

All experimental protocols involved in this study were approved by the Regulations for the Administration of Affairs Concerning Experimental Animals for the Science and Technology Bureau of China. The sample collection and experimental protocols were approved by Animal Care and Use Committee of the

Fisheries College of Jimei University (Animal Ethics **No.1067**). All animal handling and methods were performed according to the relevant guidelines.

CRediT authorship contribution statement

ZW Zhong is responsible for sampling, proposal of research scheme, carrying out the experiments, bioinformatics analysis and manuscript writing. **Y Xu, Y Feng** and **LL Ao** are responsible for sampling, cell interference experiment and quantitative analysis. **YH Jiang is** responsible for the task proposal, funding and technology, the research proposal and implementation support and critically edited the manuscript. All authors read and approved the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflicts of interest

The authors declare that they have no conflict of interest.

Consent to participate

All authors have discussed the study procedures and have been satisfied with the relevant questions, and all have agreed to participate in the study.

Availability of data and material/ Data availability

Not applicable.

Code availability

Not applicable.

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Tables

Table 1 Identity (%) of *Cv*-Nanog as well as its domains with other species.

species	Total amino acid Identity	Homeodomain Identity	Accession
Morone saxatilis	84.47%	92.06%	XP_035508194.1
Sparus aurata	80.09%	90.47%	XP_030254190.1
Perca flavescens	72.95%	90.47%	XP_028425553.1
Labrus bergylta	72.94%	88.89%	XP_020497303.1
Notolabrus celidotus	72.73%	90.47%	XP_034562206.1
Acanthochromis polyacanthus	58.26%	79.37%	XP_022054603.1
Gadus morhua	52.53%	80.95%	XP_030204181.1
Coregonus sp.'balchen'	47.21%	74.60%	CAB1349676.1
Salvelinus alpinus	47.21%	73.16%	XP_023828202.1
Salmo salar	45.02%	73.16%	XP_014015198.1
Danio rerio	42.45%	80.65%	NP_001091862.1
Oryzias latipes	56.52%	88.89%	NP_001153902.1
Mus musculus	15.41%	47.62%	AAP92157.1
Gallus gallus	16.51%	55.56%	ABK27429.1

Figures

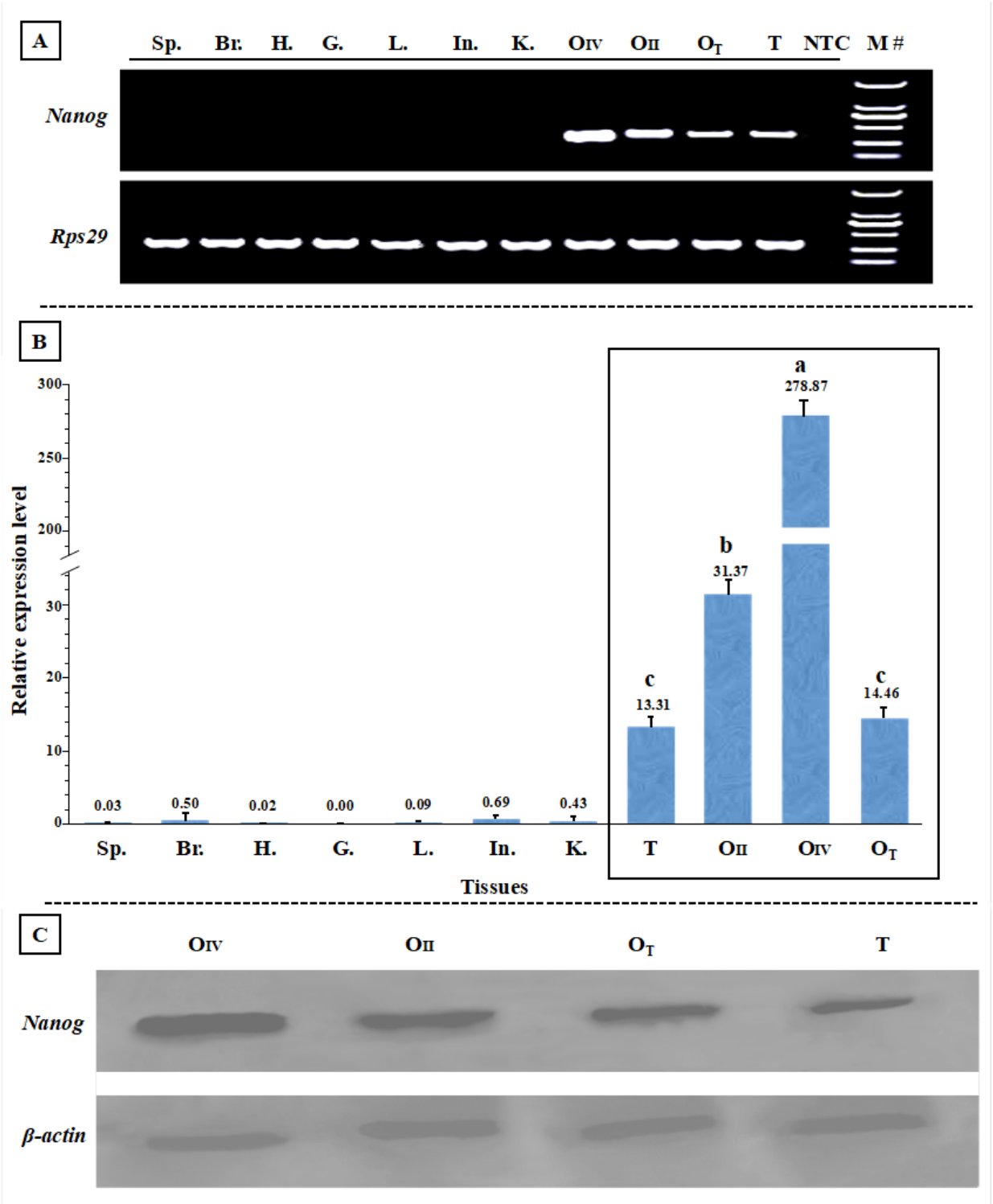

Figure 1

Expression profile of Cv-Nanog. Note: A, The expression of Cv-Nanog in different tissues by semi-RT-PCR. B, The relative expression profile of Cv-Nanog in different tissues by qRT-PCR. The data in each tissue was normalized with the value of the reference gene (Rps29), the black boxes indicate the significance levels of gonads at four different stage. C, Western blot showing the presence of Cv-Nanog in ovaries at

different developmental stages and testis of C. vrolikii with a signal at 37 kDa and β-actin was taken as a control.

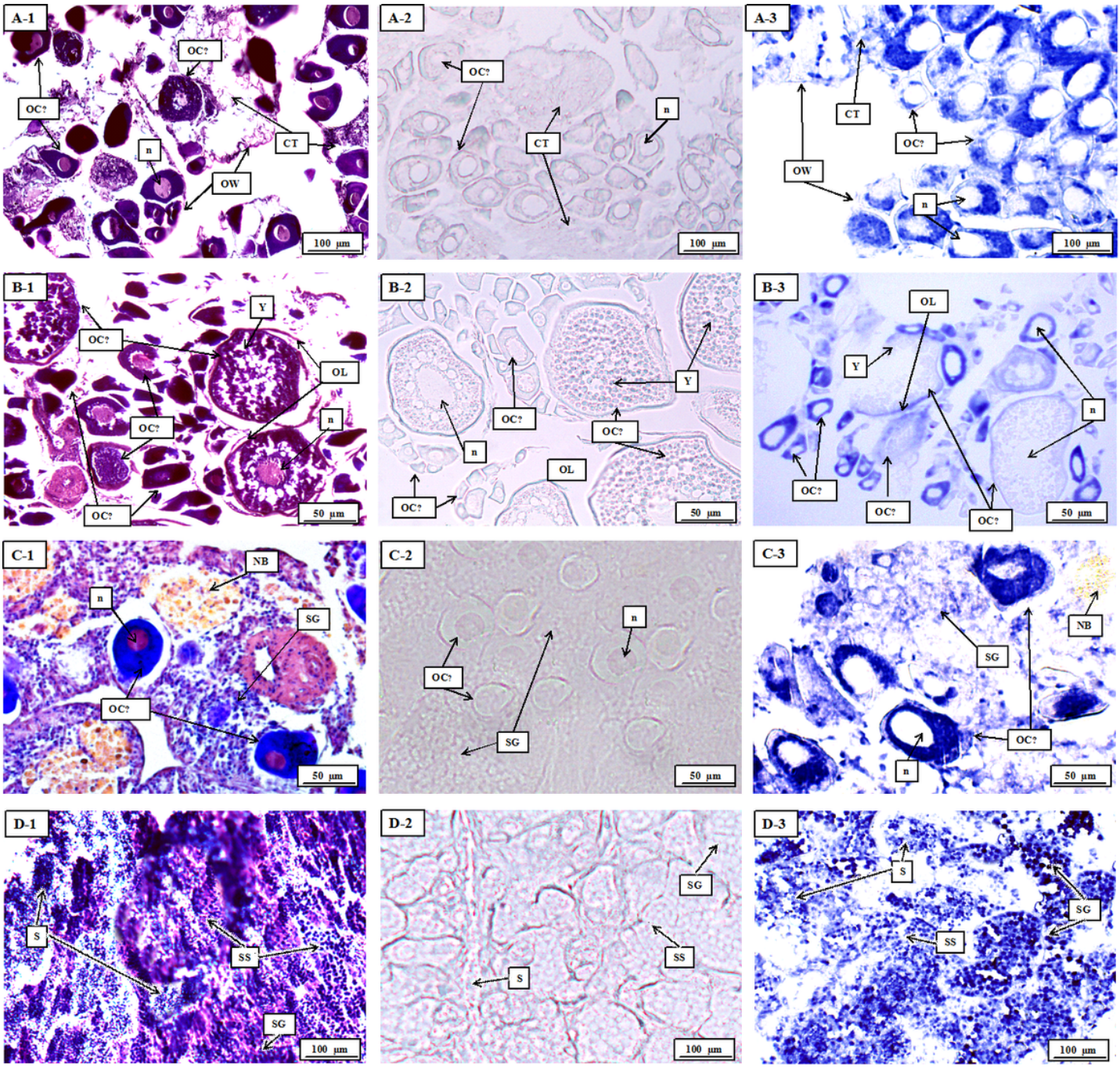

Figure 2

Expression of Cv-Nanog mRNA in gonads analyzed by ISH. Note: The histological structure of gonads at different stages in C. vrolikii by H.E. staining (A1-D1). The negative control with sense probe hybridization (A2-D2) was not stained, whereas the positive signals (A3-D3) with anti-sense probe hybridization was stained with purple or blue. A1-A3, ovary at the perinucleolus stage (O□); B1-B3, ovary at the yolk vesicle stage (O□); C1-C3, ovotestis at the hermaphroditic stage (OT); D1-D3, testis (T). OC□: the oocytes at stage □; OC□: the oocytes at stage □; OC□: the oocytes at stage □; OW: the ovarian wall; OL: the oolemma; CT: the

connective tissue; Y:the yolks; NB: the nutrient body; SG: the spermatogonia; SS:the secondary spermatocyte; S: the mature sperm; n: the nucleus.

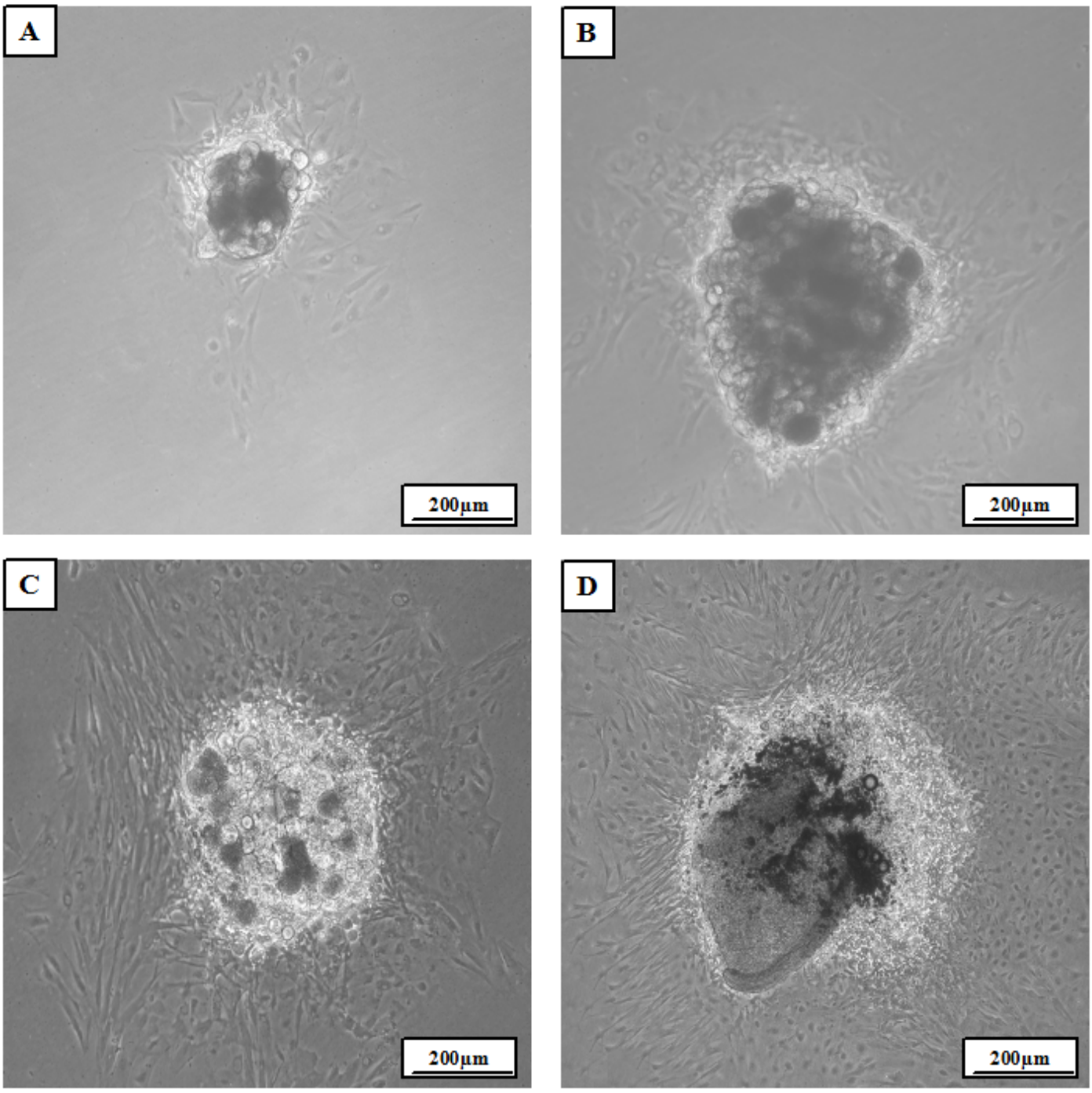

Figure 3

The cell morphologic change after overexpression of Cv-Nanog. Note: A and B show the cell morphologic change of the gonadal cells after overexpression of Cv-Nanog in 24 h, 48 h, respectively. C and D show the cell morphologic change of the gonadal cells without overexpression of Cv-Nanog in 24 h, 48 h, respectively.

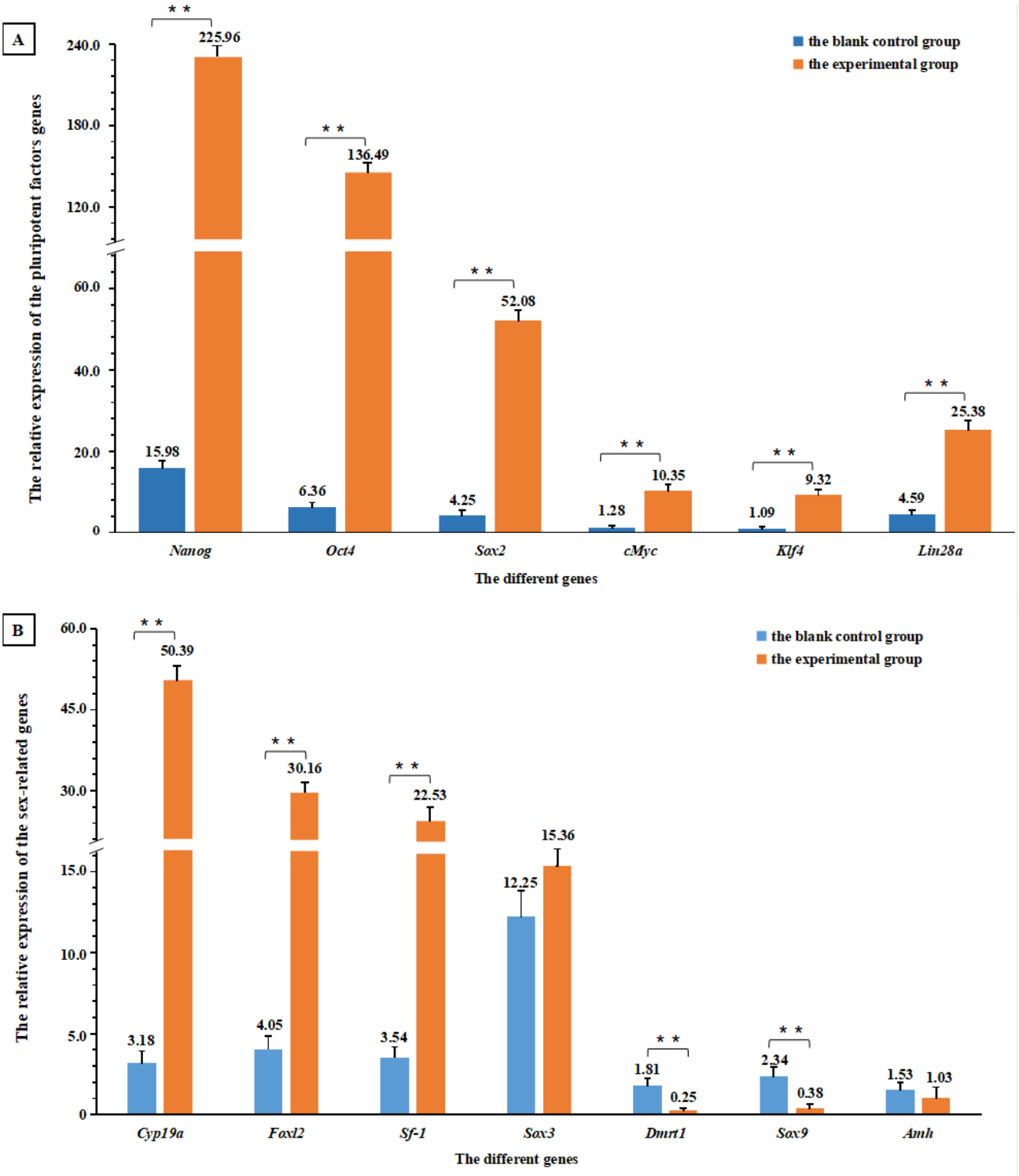

Figure 4

The expression of pluripotency factor and sex-related genes after overexpression of Cv-Nanog. Note: A shows the relative expression level of pluripotency factors after overexpression of Cv-Nanog. B shows the relative expression level of sex-related genes after overexpression of Cv-Nanog.

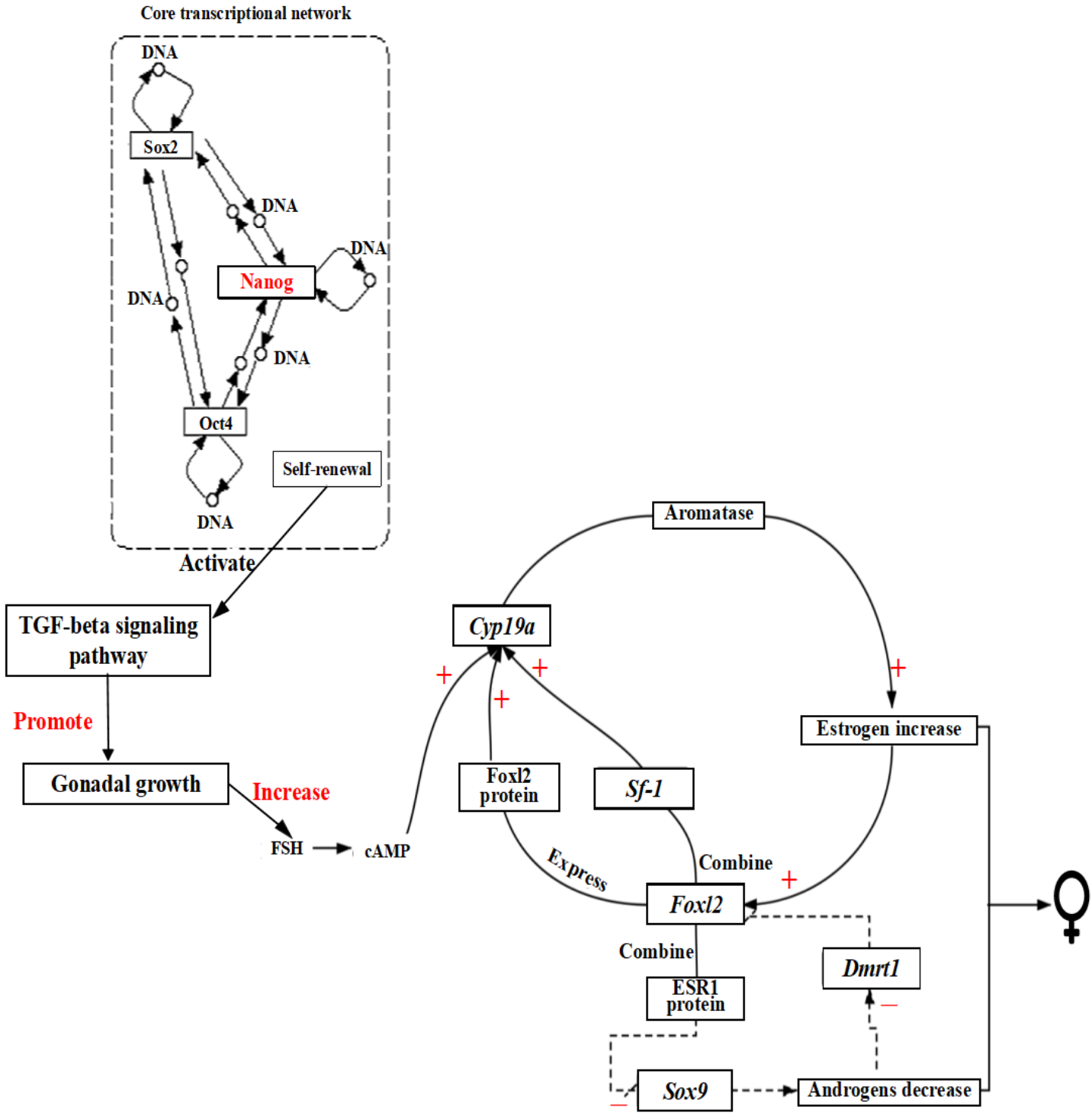

Figure 5

The presumed regulatory network of gonadal development mediated by Cv-Nanog.

Supplementary Files

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