Characterization of the Nanog gene involved in the gonadal development in pearlscale angelfish (Centropyge vrolikii)

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Abstract  The homeodomain transcription factor Nanog plays a crucial role in the embryonic and gonadal development and the maintenance of embryonic stem cells (ESCs), interacting with transcription factors such as Oct4 and Sox2 in mammals. Nevertheless, its pathways to molecular mechanisms remain unclear as to teleosts. This study investigates the role of the Nanog gene in gonadal development and sex reversal of pearlscale angelfish (Centropyge vrolikii). To understand the expression pattern of gonadal development, we identified the Nanog gene of C. vrolikii, which we named Cv-Nanog. The full-length cDNA sequence of Cv-Nanog was 2,136 bp in length and encoded a homeodomain protein of 436 amino acid residues. The gene structure and western blot prove results that Cv-Nanog was homologous to the Nanog gene of mammals. The protein sequence comparison demonstrates that the Cv-Nanog shared a high degree of similarity with orthologs from other vertebrates in the conserved homeodomain. The Cv-Nanog gene was substantially expressed in gonads, and the expression was significantly higher in the ovaries than in the testis, according to quantitative real-time PCR (qRT-PCR) and western blot analyses. In situ hybridization reveals that the transcripts were located in the cytoplasm and membrane of the oocytes in the ovaries and testes. The expression of Cv-Nanog mRNA was weak in Sertoli cells but strong 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. Simultaneously, the TGF-beta signaling pathway was activated, and the gonadal cells’ growth was promoted.

Highlights

• The Cv-Nanog gene was found to be highly expressed in the gonads.
• The expression level of Cv-Nanog was significantly higher in the ovaries than in the testis.
• In the conserved homeodomain, the Cv-Nanog shared a high degree of similarity with orthologs of other vertebrates.
• In situ hybridization revealed that the transcript was located in the germ cells of the gonads.
• After overexpression of Cv-Nanog in the gonads, the TGF-beta signaling pathway was activated, and the gonadal cells’ growth was promoted.

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pathway was activated, and the gonadal cell growth was promoted. The expression of ovary-bias genes Cyp19α and Foxl2 was upregulated, and the expression of testis-bias genes Sox9 and Dmr1 was downregulated to promote ovarian development. These results imply that the Nanog gene might play a crucial role in the process of gonadal development and sexual reversion in C. vrolikii. This study provides new insight to understand the molecular regulatory mechanism of the Nanog gene further and important clues for the future studies in gonadal development.

**Keyword**  Centropyge vrolikii; Nanog; Gonadal development; Gene expression; Sexual reversion

**Introduction**

In recent years, research on induced pluripotent stem cells (iPSCs) has become a prominent issue. The Oct4, Sox2, cMyc, and Klf4 (OSKM) genes have successfully induced the reprogramming of human somatic cells into iPSCs (Takahashi and Yamanaka 2006). Subsequently, OSKM has been regarded as a critical factor in obtaining iPSCs (Maherali and Hochedlinger 2008; Zhao et al. 2008), and with Oct4 being the only factor required for iPSC induction (Nakagawa et al. 2008). The Nanog gene, which was regarded as one of the key genes to maintain the pluripotency and self-renewal ability of the embryonic stem cells (ESCs), was discovered for the first time during the construction of a mouse ESC cDNA library (Wang et al. 2003), and it is specifically expressed in pluripotent tissues such as undifferentiated cells of cell clusters in the blastocyst stage (Mitsui et al. 2003). Subsequently, some investigations have reported that the Nanog gene may play an essential role in maintaining the pluripotency of ESCs in stem cells so that they may self-renew in the LIF/STAT3 pathway (Chambers et al. 2003; Mitsui et al. 2003). The Nanog gene has long been thought to be a marker gene of stem cells (SCs) in mammals, and knocking it down made the SCs or ESCs more likely to differentiate (Zaehres et al. 2005). Conversely, after the overexpression of the Nanog gene, the ESCs’ self-renewal ability and the expression of pluripotent factors are maintained, strengthened, and triggered, respectively (Mitsui et al. 2003; Darr et al. 2006; Silva et al. 2006).

The Nanog gene of a mouse encoded 305 amino acids, and the Nanog protein comprises of a relatively conserved homeodomain (HD), a 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 2003; Mitsui et al. 2003). Among the basic structural and functional domains, the HOX domain plays a central role in binding with DNA and interacting with proteins. In the WR region, the main functional region of the Nanog protein can interact with other pluripotent factors to maintain the self-renewal ability of ESCs in a mouse (Mullin et al. 2008; Wang et al. 2008). Unlike mammals, there is a lack of a WR domain in the carboxyl-terminal of the Nanog protein in lower vertebrates such as zebrafish (Danio rerio) and salamander (Salamandra salamandra), but its HD and the amino-terminal domain can form a dimer structure to directly regulate the pluripotency of ESCs (Dixon et al. 2010; Schuff et al. 2012).

Because of the Nanog gene’s critical role in the maintenance of pluripotency for ESCs or germ stem cells (GSCs), the expression regulation of the Nanog gene has revealed the regulatory effects of a variety of transcription factors. In the proximal promoter region of mammalian Nanog gene, there are relatively conservative Oct4, Sox2, Klf4, and Pbx1 binding sites that upregulate Nanog gene expression (Kuroda et al. 2005; Rodda et al. 2005; Chan et al. 2009). Nanog, as one of the key transcription factors regulating ESC self-renewal, is also important in iPSCs (Okita et al. 2007; Rais et al. 2013). The transcription factors, Oct4, Sox2, Nanog, and Lin28, when combined, can successfully reprogram human somatic cells to a pluripotent state with some ESC-like characteristics (Yu et al. 2007). It is worth noting that expressing two components, Nanog and Klf4, or Nanog and Bmil, for a short period of time is enough to activate the pluripotent regulatory network and reset the pluripotent state in mammalian stem cells (Moon et al. 2013; Takashima et al. 2014). So, in mammals, the Nanog gene can replace the Oct4 gene or other genes to participate in cell reprogramming. It is unknown, however, whether the Nanog gene has a similar effect in the regulation of pluripotency in fish.

Nanog is highly expressed in undifferentiated ESCs (Chambers et al. 2003) and is also found in GSCs (Yamaguchi et al. 2005), fetal testis (Kerr
et al. 2008), seminoma and breast cancer cells (Ezeh et al. 2005), and some tissues in mammals (Hart et al. 2004). The model fish medaka (Oryzias latipes) (Camp et al. 2009), as well as other teleosts such as zebrafish (Schuff et al. 2012), goldfish (Carassius auratus) (Marandel et al. 2012), and blunt snout bream (Megalobrama amblycephala) (Yu et al. 2017), have direct homologues of the mammalian Nanog gene. Unlike mammals, the Nanog gene of fish has a maternal expression pattern that can be detected in unfertilized eggs and is highly expressed until the blastocyst stage. Furthermore, the Nanog gene is clearly expressed in the adult gonads of teleost fish during gonadal development (Wang et al. 2011), and the expression of the Nanog gene has also been detected in the liver of blunt snout bream (Yu et al. 2017), suggesting that it may be relevant to the existence of adult stem cells. The role of Nanog gene in gonadal development of teleost fish is worthy to further study and exploration.

The pearlscale angelfish (Centropyge vrolikii), commonly known as the black tail angelfish, mainly distributed in the Indian Ocean and the Pacific Ocean near the coral reefs, is a unique marine ornamental species with a tremendous market potential (Dibattista et al. 2016; Fernandez-Silva et al. 2018). Currently, C. vrolikii is obtained primarily through wild fishing; due to increased market demand and environmental destruction, the natural catch of C. vrolikii has decreased year by year, and artificial breeding is unavoidable. However, because C. vrolikii is a protogynous hermaphrodite with natural sexual reversion, the disadvantage of a long breeding cycle and difficulty spawning has become the main barrier to breeding (Zhong et al. 2021). As a result, it is critical to investigate the mechanism of molecular regulation in gonadal development of C. vrolikii. Previously, we used RNA-Seq technology to screen for the differentially expressed genes (DEGs) in C. vrolikii gonads at various stages (Zhong et al. 2021). However, the Nanog gene expression in fish, designated as Cv-Nanog, has received little attention at the molecular level. The regulatory network of Nanog has been extensively studied in mammals, but the molecular mechanism of the regulatory network in fish has not been reported. The present study aimed at identifying the Cv-Nanog and understanding the regulatory network of Cv-Nanog. Our findings will provide a new perspective on gonadal development and artificial breeding in small sea angelfish.

**Materials and methods**

Sample collection and preparation of tissue sections

C. vrolikii were obtained from the Aquatic Product Experiment Center of Fisheries College Jimei University (Xiamen, China). The gonads were collected from four developmental stages, including the ovary at the yolk vesicle stage (OIV), ovotestis at the hermaphroditic stage (OIT), ovary at the yolk vesicle stage (OIV), and pure testis (T), according to our previously published peer-reviewed article (Zhong et al. 2021).

One part of gonads (0.5 cm³) was quickly fixed in 4% paraformaldehyde (PFA) at 4 °C for 24 h for further use, while the rest of gonads and other tissues (kidney, liver, spleen, brain, gill, eye, intestine, and heart) were preserved in RNAlater (Ambion) and stored at 4 °C for 24 h before being transferred to −80°C for storage until RNA extraction and qRT-PCR.

The gonads (in PFA) were dehydrated, decolorized, waxed, and sectioned continuously into 6 μm. After that, the sections were stained with hematoxylin–eosin (H.E), sealed with neutral gum, and observed on a microscope.

Molecular cloning and analysis of Cv-Nanog

Primer 5.0 software was used to create the primers (P-Nanog-F/-R, Table S1 in Supplementary), which were based on RNA-Seq data from the gonad transcriptome of C. vrolikii. The polymerase chain reaction (PCR) was used to clone a partial fragment of Cv-Nanog using the cDNAs of ovaries as a template for amplification. Primer 5.0 was used to design the nested PCR primers of Cv-Nanog to clone the 5′ and 3′ regions (Table S1 in Supplementary). The total RNA of each tissue was extracted according to the instructions, and the first cDNA strand was synthesized from 3.0 μg total RNA samples. As a PCR template, the cDNA was diluted 10 times using DEPC water, and the following was the PCR amplification procedure: 94 °C for 4 min; 35 cycles of 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 1 min; followed by 72 °C for 10 min. The PCR products were separated by agarose gel electrophoresis.
and then recovered using the gel Recovery Kit (Promega). DNA connection kit (TAKARA) was used to connect the target fragments to a 19-T vector, which was subsequently converted into DH5α competent cells. The recombinant product was coated on a solid medium with ampicillin and cultured at 37 °C for 16 h. The monoclonal cell was then identified using the M13 universal primer. To guarantee amplification accuracy, the positive clones were grown, cultured, and sequenced.

The National Centre for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to conduct and analyze nucleotide and protein sequences of Cv-Nanog. The Expasy ProtParam Tool (http://web.expasy.org/protparam/) was used to calculate the theoretical amino acid composition, PI and MM. N-glycosylation sites (N-X-S/T) were predicted with NetNGlyc1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and phosphor- ylation 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). A STRING database (https://string-db.org/cgi/input.pl) was used to show how the Nanog protein interacts with other proteins. The phylogenetic tree was constructed using the bootstrap neighbor-joining (NJ) method of MEGA 7.0 (http://www.megasoftware.net), and the bootstrap values were duplicated 1,000 times.

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 for reference genes referred to a previously published peer-reviewed publication that used Rps29 as a tissue expression reference gene (Zhong et al. 2021). Each sample’s reverse transcription products were suitably diluted as templates for qRT-PCR, and each sample was tested with three technical duplicates. For the qRT-PCR analysis, SYBR Green Master Mix (Vazyme) was employed, and the relative fold change of the gene expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. All of the primers are shown in Table S1 in Supplementary.

In situ hybridization

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

Western blot

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

For SDS-PAGE, firstly, the mixture (protein samples and 5×SDS-PAGE loading buffer, 4:1) was boiled for protein denaturation at 100°C for 15 min. Then, the mixture and prestained protein marker were added to the 10% separation gel wells, respectively. After electrophoresis at 80 V for 30 min, the voltage was adjusted to 120 V for roughly 50 min, until the indicator reached the bottom of the gel. Secondly, the proteins were transferred from the gel to a PVDF membrane using a membrane transferring apparatus under 200 mA for 90 min. After transfer, the PVDF membrane containing proteins was washed with PBS or PBST three times for 5 min. It was then placed in a small box with 5% BSA blocking solution (diluted
with 1×PBS) and incubated at 37°C for 1 h. Thirdly, the membrane was washed with PBS or PBST three times for 5 min before being put 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°C overnight. On the second day, the membrane was washed with PBS three times for 10 min each time, then faced up by adding 2 mL of a chromogenic agent until significant brown coloring bands were visible and observed under e-Blue touch imager.

Liposome transfection and cell culture

The fish were washed and immersed in a bucket containing 1‰ commercial hypochlorous acid disinfectant for 1 h, before being anesthetized with 3–4 drops of eugenol (approximately 0.1‰ of water volume). After that, the gonads were thoroughly dissected carefully and washed three times in PBS containing amphotericin B and double-antibody. The tissues were chopped into small pieces (about 1 mm³) and washed three times with PBS. The tissues were seeded evenly in a six-well cell culture plate, infiltrated with 1 mL cell culture medium (L-15 containing 15% FBS, EGF, HGF, β-FGF, CMC, N-AG and 2-Me), and cultured at 27°C for 2 days. On the third day, 2 mL cell culture medium was added for continued culture.

The CDS of the Nanog gene without the stop codon was inserted into the pEGFP-N1 vector to obtain the overexpression vector (pNanog-N1), and the double digestion primers (primers: O-Nanog-F2/-R2) were designed using the website (https://crm.vazyme.com/cetool/singlefragment.html), as described in our earlier experiment (Xu et al. 2022). 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. Referring to the user manual of Lipofectamine™ 2000, solution A and solution B were prepared in a new 1.5-mL EP tube. In a 6-well plate, the dose for each well is as follows: solution A: 2 μg pNanog-N1 plasmid was added to 250 μL Opt I MEM medium and allowed to stand at room temperature for 5 min; solution B: 10 μL Lipo2000 was added to 250 μL Opt I MEM medium and allowed to stand at room temperature for 5 min. The solutions A and B were combined and allowed to rest at room temperature for 20 min. Then, the mixture was added to each 6-well plate, respectively, gently mixed to ensure uniform contact with the cells and tissues, and cultured in a CO₂ incubator. After 6 h of interaction with cells (tissues), the liposome-DNA complex was replaced with a full medium containing 15% FBS for culture. At 24 h and 48 h after transfection, the cells were observed, and the cell growth was recorded. The RNA of the gonads (cells) after transfecting was extracted and reverse transcribed into cDNA to identify the pluripotency factor, and the sex-related genes were both detected by qRT-PCR. All of the primers are shown in Table S1 in Supplementary.

Statistical analysis

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

Result

Cloning and characterization of Cv-Nanog

The 3′ untranslated region (3′ UTR) and 5′ UTR were obtained by 3′ and 5′ RACE PCR, and the double digestion primers (primers: O-Nanog-F2/-R2) were designed using the website (https://crm.vazyme.com/cetool/singlefragment.html), as described in our earlier experiment (Xu et al. 2022). 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. Referring to the user manual of Lipofectamine™ 2000, solution A and solution B were prepared in a new 1.5-mL EP tube. In a 6-well plate, the dose for each well is as follows: solution A: 2 μg pNanog-N1 plasmid was added to 250 μL Opt I MEM medium and allowed to stand at room temperature for 5 min; solution B: 10 μL Lipo2000 was added to 250 μL Opt I MEM medium and allowed to stand at room temperature for 5 min. The solutions A and B were combined and allowed to rest at room temperature for 20 min. Then, the mixture was added to each 6-well plate, respectively, gently mixed to ensure uniform contact with the cells and tissues, and cultured in a CO₂ incubator. After 6 h of interaction with cells (tissues), the liposome-DNA complex was replaced with a full medium containing 15% FBS for culture. At 24 h and 48 h after transfection, the cells were observed, and the cell growth was recorded. The RNA of the gonads (cells) after transfecting was extracted and reverse transcribed into cDNA to identify the pluripotency factor, and the sex-related genes were both detected by qRT-PCR. All of the primers are shown in Table S1 in Supplementary.
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).

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. For example, 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%), Percia 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).

Protein–protein interactions for Nanog

The neighborhood, gene fusion, co-occurrence, co-expression, and homology scores with the interacting proteins were all taken into account. The Nanog protein was a principal member of the NOS triad, which was crucial responsible for stem cell pluripotency and maintenance. Protein–protein interacting networks of Nanog and other factors (Table S3 in Supplementary; see Fig. S4 in Supplementary) involving pluripotency were 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), and cMyc (a transcription factor that binds DNA in a non-specific manner) (Table S3 in Supplementary).

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          |
| Percia 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_01091862.1           |
| 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              |
Expression of Cv-Nanog

The expression of *Cv-Nanog* in various tissues of *C. vrolikii* was analyzed by semi-RT-PCR (Fig. 1A) and qRT-PCR (Fig. 1B). The *Cv-Nanog* was found to be highly expressed in the ovaries. Furthermore, the highest expression level occurred in the ovary at the yolk vesicle stage (OIV) (*p* < 0.01), which was higher (about 8.9-fold) than in the ovary at the perinucleolus stage (OII), was much higher (about 19.3-fold and 20.9-fold) than in the gonads at hermaphroditic stage (OT) and testes, respectively. On the other hand, the expression of *Cv-Nanog* was low in the remainder 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 around 37 kDa in the protein of gonads at each stage, which was identical 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 OIV, then gradually decreased in the OII, OT, and T (Fig. 1C).

Distribution of Cv-Nanog

In situ hybridization on 5-μm-thick paraffin sections was performed to identify the distribution pattern of *Cv-Nanog* in different developmental gonads. The ovary of OII stage was dominated by the oocytes at stage II with small cell volume and no yolks (Fig. 2A-1), while the ovary of OIV stage was dominated by oocytes at stage IV with large volume and rich yolks (Fig. 2B-1). Male and female germ cells coexisted in the gonads at OT stage (Fig. 2C-1). By ISH, *Cv-Nanog* mRNA expression was mainly concentrated on the cytoplasm and membrane of the oocytes. At OII stage, the positive signal was mainly detected in the cytoplasm of the oocytes at stage II (Fig. 2A-3). At the OIV stage, the positive signals were also detected on the membrane of the oocytes at stage IV (Fig. 2B-3). At OT stage, *Cv-Nanog* mRNA expression was higher in oocytes than sperm (Fig. 2C-3). There were several seminiferous lobules in the testis, and each lobule contained different developmental stages of male germ cells (Fig. 2D-1). The results showed that *Cv-Nanog* mRNA expression was weakly expressed in Sertoli cells, but highly expressed in sperm (Fig. 2D-3).

Expression of sex-related genes after Nanog overexpression

During the 2 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 tenser (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. 4A). In addition, the expression of sex-related genes also changed in varying degrees. The expression of the ovary-related gene *Cyp19a* increased significantly, and *Foxl2* and *sf-1* also increased significantly (Fig. 4B). The expression of testis-related genes *Dmrt1* and *Sox9* decreased significantly (Fig. 4B). 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 the TGF-beta signaling pathway was activated and the gonadal growth was promoted (Fig. 5).

Discussion

The role of the pluripotent gene *Nanog* in embryonic development and sustaining pluripotency in ESCs, as well as the regulatory networks, have been researched extensively since its discovery and isolation in mouse ESCs (Chambers et al. 2003; Darr et al. 2006; Mitsui et al. 2003; Wang et al. 2003). From then on, the *Nanog* gene has also been reported in birds (Canon et al. 2006; Laval et al. 2007) and certain model species, including 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 has been known about the *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...
Fig. 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.
described, in order 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. The *Cv-Nanog* protein, like medaka (Wang et al. 2011), zebrafish (Theunissen et al. 2011), and blunt snout bream (Yu et al. 2017), has a conserved HOX domain, which is located at 222–284 (Fig. 2). In addition, the HOX region also contained a conserved YKQVKTWFQN motif, similar to that observed in human Nanog, that was speculated to represent a

Fig. 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 were stained with purple or blue. A1–A3 Ovary at the perinucleolus stage (OII); B1–B3 ovary at the yolk vesicle stage (OIV); C1–C3 ovotestis at the hermaphroditic stage (OT); D1–D3 testis (T). OCII, the oocytes at stage II; OCIII, the oocytes at stage III; OCIV, the oocytes at stage IV; 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; GV, the germinal vesicle; n, the nucleus
nuclear localization signal (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. 2008). Some studies also pointed out that the WR region played an important role in interacting 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 could play an effective role in the process of cell reprogramming in mammals, birds, and teleost fish (Theunissen et al. 2011), indicating that it has a conserved function in vertebrates. The HOX domain,
Fig. 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.
which is an essential component structure of Nanog, can detect and bind particular DNA sequences in order to perform its function (Jauch et al. 2008). Except for the conserved Hox domain, the conservation of other sequences was rather low, according to multiple sequence alignment. The full-length Cv-Nanog protein has a lower sequence identity (16.5%) than that of the mouse Nanog (Table 1). In zebrafish, the Dr-Nanog sequence identity was much lower (13%) compared to that in mouse despite the fact that they were functionally conserved (Theunissen et al. 2011). It is speculated that the Nanog gene has a high rate of evolution and a rich range of properties. 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 findings revealed that the HOX domain of Nanog was exceedingly conservative in evolution and fundamental in function.

Gonadal development is crucial to the growth and reproduction of fish. The Nanog gene was found to play essential roles in embryogenesis, gonadal development, and maintaining the pluripotency and self-renewal ability of ESCs in vertebrates during a slew of research (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 the transcript of Cv-Nanog was expressed explicitly in gonads at different developmental stages with much higher in ovaries than that in testes, 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 also been described (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, testes and ovaries (Clark et al. 2004), and ovarian cancer cells (Hoei-Hansen et al. 2005, 2007). In mice, the expression of Nanog is significantly higher than that in the heart, kidney, spleen, liver, and differentiated cells (Hart et al. 2004; Ventea et al. 2012). In medaka, the expression of Nanog is exhibited in the gonads, predominantly in pluripotent cells (Camp et al. 2009; Wang et al. 2011). C. vrolkii is a naturally reversed fish, the mark of the beginning of the gonad reversal is the appearance of the male germ cells in the ovary, known as hermaphrodite, followed by further differentiation of the male germ cells, and eventually, the gonad is turned 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 IV > O II > O T > T in turn (Fig. 5). It is speculated that Nanog was liable to be indispensable in the process of 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 unclear 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 II (Fig. 2A1–A3), and it was only localized in the membrane of the oocytes with no positive signal in yolk granules at stage IV (Fig. 2B1–B3). In O T stage, the Nanog RNA expression was distributed in both oocytes and sperm (Fig. 2C1–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. 2D1–D3). The expression of Cv-Nanog in the gonadal germ cells was comparable to mouse and chicken (Canon et al. 2006; Lavial et al. 2007; Mitsui et al. 2003; Yamaguchi et al. 2005). Inconsistently, the Nanog RNA expression is present in spermatogonia or oogonia in medaka (Wang et al. 2011) and the early stage of oocytes in the ovary and spermatogonia, spermatocytes, and spermatids in the testis in blunt snout bream (Yu et al. 2017). According to the current western blot study, Cv-Nanog protein was most strongly expressed in O IV, then gradually decreased in O II, O T, and T (Fig. 5C). The results of western blot analysis were compatible with those of qRT-PCR, suggesting that the Nanog gene was expressed in gonads with highly in ovaries. Thus, the potentiating presence of Cv-Nanog mRNA and protein in the ovaries could be argued as its maternal inheritance, which agrees with the teleost observations (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 (fivefold), cMyc (twofold), and Sall4 (fivefold) 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 gonad cell lines (LYCO and LYCT) of large yellow croaker ( Larimichthys crocea ) (Xu et al. 2022). 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. Furthermore, 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 gonadal development. Then, the follicle-stimulating hormone (FSH) was promoted, the cAMP increased, and the expression of *Cyp19a* and *Foxl2* was upregulated, the expression of *Sox9* and *Dmrt1* was downregulated, and eventually the ovarian development was promoted. The high abundance of the sex-related genes in the gonads suggests its participation in maternal inheritance and 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*, and 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.

### Conclusion

In summary, this study provided the full-length cDNA sequence of the *Nanog* gene in *C. vrolikii*. We deduced that *Cv-Nanog* is the ortholog of *Nanog* of mammalian and teleost by the gene structure, the protein alignment, the phylogenetic tree, the expression pattern, and the western blot analysis. 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 demonstrate that the maternal inheritance, expressed during gonadal development, particularly high in ovaries.ISH reveals that *Cv-Nanog* RNA exhibited a sustained expression pattern in ovaries, while in testis, *Cv-Nanog* RNA exhibited specific in spermatogonia. *Nanog* overexpression experiment in cells showed that *Cv-Nanog* might 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.

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### Author contribution

ZW Zhong is responsible for sampling, the 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 experiments, 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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### Availability of data and material

Not applicable.

### Code availability

Not applicable.

### 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 the 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.

**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.

**Consent for publication** All authors read and approved the final manuscript for publication.

**Conflict of interest** The authors declare no competing interests.

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