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Short Communication

Identification and characterization of a *nanog* homolog in Japanese flounder (*Paralichthys olivaceus*)

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ABSTRACT

The homeodomain-containing transcription factor *nanog* plays a key role in maintaining the pluripotency and self-renewal of embryonic stem cells in mammals. Stem cells offered as a significant and effective tool for generation of transgenic animals and preservation of genetic resources. The molecular genetic organization and expression of *nanog* gene in marine fish have not been reported yet. In this study, we isolated and characterized the flounder *nanog* gene as a first step towards understanding the mechanism of the plurpotency of fish stem cells and develop a potential molecular marker to identify the stem cells *in vivo* and *in vitro*. Phylogenetic, gene structure and chromosome synteny analysis provided the evidence that *Po-nanog* is homologous to the mammalian *nanog* gene. Protein sequence comparison showed that flounder Nanog shared low similarity with other vertebrate orthologs except for a conserved homeodomain. Quantitative RT-PCR analysis showed that flounder *nanog* was maternally expressed, and the transcripts were present from the one-cell stage to the neurula stage with the peaking at blastula stage. Whole mount *in situ* hybridization analyses demonstrated that the transcripts were present in all blastomeres of the early embryo. Tissue distribution analysis indicated that *nanog* was detectable only in gonads. Further, the expression was significantly high in ovary than in testis. *In situ* hybridization revealed that the transcripts were located in the cytoplasm of the oogonia and oocytes in ovary, only in the spermatogonia but no spermatocytes or spermatids in testis. The promoter region was also analyzed to have several basal core promoter elements and transcription factor binding sites. All these results suggest that *Po*-Nanog may have a conservative function between teleosts and mammals.

1. Introduction

Nanog is a newly found homeodomain-containing transcription factor distinct from the other homeobox genes and has been extensively analyzed in mammalian in the last decade. Nanog is well known to be involved in maintaining the pluripotency and self-renewal of embryonic stem cells together with other transcription factors such as Oct4 and Sox2 (Boyer et al., 2005; Chambers and Tomlinson, 2009; Chambers et al., 2003; Lavial et al., 2007; Mitsui et al., 2003). Nanog also has a conserved capacity in reprogramming the somatic cells back into the pluripotent state (Moon et al., 2013; Silva et al., 2009; Theunissen et al., 2011) despite its low sequence identity.

In mammals, *nanog* has been studied and considered as a pluripotency marker expressed in the inner cell mass (ICM) of the blastocyst *in vivo* and strictly in the "stem" property cells *in vitro* but down-regulated in the differentiated cells (Hart et al., 2004; He et al., 2006; Yamaguchi et al., 2005). In other higher animals, chicken *nanog* is expressed in the primordial germ cells of the embryo and the function in cESC is identical to mammals (Canon et al., 2006; Lavial et al., 2007). Recent studies have revealed that *nanog* also exists in the lower vertebrate and mainly focused on the model teleost fish. Different from the mammals, the fish *nanog* is maternal inherited and can be detected in the gonads besides the early development embryos. In medaka, Camp et al. (2009) first characterized the expression profile of medaka *nanog* and demonstrated its role in regulating proliferation of the developing embryo. Later, Wang et al. (2011) identified *nanog* to be one of the pluripotency genes in medaka and pointed out its specific expression in the spermatogonia of the adult testis. In zebrafish, it is revealed that *nanog* regulates the blastomere division and germ layer patterning (Tian et al., 2011). Moreover, the zebrafish *nanog* is found to be crucial for survival of early embryos and can act as a substitution in promoting proliferation and LIF-independent self-renewal of mouse ES cells (Schuff et al., 2012). Therefore, these studies have led to the assumption that *nanog* may play a conserved role between mammals and teleosts in spite of its rapid evolution in vertebrate.

The Japanese flounder, *Paralichthys olivaceus*, is a commercial important flatfish species farmed throughout the coastal of Northeast China for its good taste and nutritive value. Over the last decade, great efforts had been made in breeding of fast-growing and anti-disease varieties, and the work mainly focused on traits related gene screening and

Abbreviations: ESC, embryonic stem cell; HD, homeodomain; ICM, inner cell mass; qRT-PCR, quantitative real-time PCR; RACE, rapid amplification of cDNA ends; TF, transcription factor; TIS, transcription initiation site; TSS, transcription start site; UTR, un-translated region.

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molecular marker-assisted breeding (Coimbra et al., 2003; Fan et al., 2010; Fuji et al., 2007; Hwang et al., 2011; Song et al., 2012; Xu et al., 2008b; Zhong et al., 2008a). Obviously, it is necessary to do some research in order to protect the genetic resources of the fine varieties. Successful cryopreservation would help a lot within this context. Although sperm cryopreservation has been a routine process, the oocytes and embryos cryopreservation remains problematic in fish (Edashige et al., 2006; Guan et al., 2008; Zhang et al., 2003). Another promising tools for genetic resources conservation are the fish embryonic and germ stem cells (Alvarez et al., 2007; Xu et al., 2010). Particularly, the fish ESCs possess the ability of replicate indefinitely and can differentiate into all three layers of the embryo. Furthermore, fish ESCs can also help to generate the transgenic animals with site-directed gene mutations (Hong and Schartl, 2006). Generally, the fish ESCs provide a useful tool in conservation and aquaculture. Up to now, it is encouraged that several fish ES cell lines have been attempted and successfully established under a feeder-free condition by using the blastula stage embryos (Alvarez et al., 2007; Béjar et al., 2002; Chen et al., 2003; Hong and Schartl, 1996; Parameswaran et al., 2007; Yi et al., 2009). However, the information of the related approach and molecular mechanism is limited in *P. olivaceus*. The aim of this study is to identify the mammalian stem cell marker gene orthologue in Japanese flounder so as to lay a foundation for the further investigation.

In our current study, we first cloned the genomic sequence of the transcription factor *nanog* from Japanese flounder, quantified the expression levels in the early development stages and different tissues, and investigated the subcellular localization of the *nanog* transcript in the embryos and adult gonads. Using protein comparison, phylogenetic and gene structure analysis, we demonstrated that flounder *nanog* is orthologue of mammalian *nanog*. Additionally, potential regulatory motifs of the promoter analyses were implemented to provide some information for further study on the function of this gene in teleost fish *P. olivaceus*.

2. Materials and methods

2.1. Fish and embryos collections

The samples were obtained from a commercial hatchery in Haiyang city, China. Six healthy adults (three females and three males) were randomly sampled and over anesthetized with MS-222. Heart, liver, spleen, kidney, whole brain, intestine, gonads (ovary and testis), gill and skeletal muscles tissues were collected and frozen immediately in liquid nitrogen, then stored at −80 °C until use. Fertilized eggs were obtained by artificial fertilization and incubated at 17 °C in clean sea water with aeration. The embryonic stage of each sample was determined via microscopy. Twelve embryonic stages (unfertilized egg, 1 cell, 2 cells, 4 cells, 8c ells, 16 cells, 32 cells, multi-cells, morula, high blastula, low blastula, early gastrula, mid-gastrula, late gastrula, neurula, heart-beating, tail-bud, hatching stage) were selected. Two pools of embryos that came from mix family were collected separately at particular developmental stage with a nylon net (100 mesh). The embryo samples were immersed into 1 ml RNAwait liquid (Solarbio, SR0020, Shanghai), then stored at −80 °C until use. The staged embryos and gonads (ovary and testis) samples used for in situ hybridization were fixed immediately in 4% paraformaldehyde–PBS (4% PFA) overnight at 4 °C, dehydrated in a gradient increasing methanol and stored in 100% methanol at −20 °C. The embryos were microscopically dissected to remove the envelopes before dehydrated.

2.2. Total RNA and genomic DNA isolation

Total RNA was isolated separately from the sampled tissues or embryos using Trizol reagent (Invitrogen) according to the manufacturer's instructions. DNA contamination was removed by DNase I (Takara) treatment, and verified by PCR using β-actin gene specific primers spanned the intron. Genomic DNA was extracted from the muscle tissue via the phenol-chloroform procedure (Sambrook et al., 1989). The quantity and quality of total RNA and genomic DNA were determined by agarose gel electrophoresis and Nanophotometer Pearl (Implen GmbH, Munich, Germany).

2.3. Cloning and sequencing of P. olivaceus nanog gene

Total RNA used for obtaining *nanog* cDNA was extracted from blastula stage embryos. One microgram of total RNA was reverse transcribed using M-MLV reverse transcriptase (RNase H$^-$) (Takara, Dalian, China) and random primer to obtain the first-strand cDNA used for cloning the central part sequence of the gene. Through searching the transcriptome sequencing data of *P. olivaceus* constructed by our laboratory (unpublished), we found two short sequences that had high identities to *Oryzias latipes nanog* gene. After blast on the NCBI, there is a gap about 200 bp between these two partial fragments. Using two degenerate primers (Nanog-Fw/Rv) designed to span these two parts, another partial fragment (an overlapping sequence) was obtained and sequenced. We assembled these three parts and got a larger central part of *Po-nanog* gene. The remaining unknown regions of the cDNA were obtained by 5′ and 3′ RACE reactions with SMART RACE PCR Amplification kit (Clontech, CA, USA) using the gene specific primers Nanog-5′Rv and Nanog-3′Fw (Table 1). Specific primers Nanog-w-Fw and Nanog-w-Rv (Table 1) which are flanking the entire open reading frame were designed to obtain the genomic DNA sequence and to confirm the resulted full-length coding sequence. The exon and intron boundaries were determined by alignment of the obtained cDNA sequence with the genome sequence generated above. The promoter region of *Po-nanog* was amplified by using the Genome Walking kit (Takara), and the primers Nanog-pro-SP3/2/1 (Table 1) were designed according to the instruction. All the amplified PCR products were separated by agarose gel electrophoresis, purified, cloned into pMD18-T (Takara, Dalian, China) and sequenced.

2.4. Quantitative real-time PCR (qRT-PCR)

The RNA samples from developmental embryos and tissues of six healthy adults were analyzed by qRT-PCR. Unfertilized eggs/embryos at different developmental stages were pooled for qRT-PCR with each pool containing 50 eggs/embryos. Two pools were prepared for each stage for replicates. The cDNA was generated by the method mentioned above. Primers for real-time PCR Nanog-RT-Fw/Rv (Table 1) were designed across exon–intron borders to avoid amplification of genomic

Table 1
Primers used in this study.

Primers	Sequence(5′-3′)	T_m (°C)	Usage
Nanog-Fw	AGCAGGGAAGGAGGTTTG	54.5	Fragment PCR
Nanog-Rv	CCGATAAGGTGGGATGTG	53.6	Fragment PCR
Nanog-5′Rv	TCCCTGCCACAGCGAATG	60.6	5′RACE PCR
Nanog-3′Fw	TCTTCCCAGAACTCAATGCC	57.3	3′ RACE PCR
Nanog-w-Fw	ATCTTGGCAACAATCCTC	49.6	Full-length PCR
Nanog-w-Rv	GGACCCATCGAGTATCAC	49.2	Full-length PCR
Nanog-pro-SP3	CGATGTGAACGATGGAGGATTG	62.6	Genome walking
Nanog-pro-SP2	ACGAGGGGTTGTACTTGTAGCC	60.1	Genome walking
Nanog-pro-SP1	AGGTGGTTCCCGTGGTTCTGC	65.7	Genome walking
Nanog-RT-Fw	CGCACACCTCACCAGACTCAT	60.0	q RT-PCR
Nanog-RT-Rv	CCTGTCACGCACCTCACTTTC	60.0	q RT-PCR
18S-RT-Fw	GGTAACGGGGAATCAGGGT	60.0	q RT-PCR
18S-RT-Rv	TGCCTTCCTTGGATGTGGT	60.0	q RT-PCR
Ubce-RT-Fw	TTACTGTCCATTTCCCCACTGAC	60.0	q RT-PCR
Ubce-RT-Rv	GACCACTGCGACCTCAAGATG	60.0	q RT-PCR
Nanog-SRT-Fw	GCCTCATCCATTCGCTGTG	59.3	Semi-RT-PCR
Nanog-SRT-Rv	GCTGGTTGTAATGCTCCTTGA	57.9	Semi-RT-PCR
β-Actin-Fw	GAGATGAAGCCCAGAGCAAGAG	58.0	Semi-RT-PCR
β-Actin-Rv	CAGCTGTGGTGGTGAAGGAGTAG	58.0	Semi-RT-PCR
Nanog-ISH-Fw	GAGCATTACAACCAGCACAT	53.2	ISH-probe
Nanog-ISH-Rv	GCGAAATACATTCACGAAAG	53.6	ISH-probe