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Molecular and expression characterization of a *nanos1* homologue in Chinese sturgeon, *Acipenser sinensis*

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ABSTRACT

The *nanos* gene family was essential for germ line development in diverse organisms. In the present study, the full-length cDNA of a *nanos1* homologue in *A. sinensis*, *Asnanos1*, was isolated and characterized. The cDNA sequence of *Asnanos1* was 1489 base pairs (bp) in length and encoded a peptide of 228 amino acid residues. Multiple sequence alignment showed that the zinc-finger motifs of Nanos1 were highly conserved in vertebrates. By RT-PCR analysis, *Asnanos1* mRNAs were ubiquitously detected in all tissues examined except for the fat, including liver, spleen, heart, ovary, kidney, muscle, intestines, pituitary, hypothalamus, telencephalon, midbrain, cerebellum, and medulla oblongata. Moreover, a specific polyclonal antibody was prepared from the *in vitro* expressed partial *As*Nanos1 protein. Western blot analysis revealed that the tissue expression pattern of *As*Nanos1 was not completely coincided with that of its mRNAs, which was not found in fat, muscle and intestines. Additionally, by immunofluoresence localization, it was observed that *As*Nanos1 protein was in the cytoplasm of primary oocytes and spermatocytes. The presented results indicated that the expression pattern of *Asnanos1* was differential conservation and divergence among diverse species.

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1. Introduction

The *nanos* gene encoding an RNA binding protein containing a zinc-finger motif was essential for germ line development in diverse organisms, although the regulated processes varied among species and between different homologs. The *nanos* gene was first identified as a maternal effect gene in *Drosophila* required for abdomen formation (Lehmann and Nusslein-Volhard, 1991). Further study indicated that the single *nanos* homolog was involved in germ cell migration, suppression of somatic cell fate in the germ line and maintenance of stem cell self-renewal (Asaoka-Taguchi et al., 1999; Hayashi et al., 2004; Wang and Lin, 2004). However, three *nanos* homologs (*nos-1*, *nos-2* and *nos-3*) had been identified in *C. elegans*. *Nos-1* and *nos-2* were required for the incorporation of PGCs into the somatic gonad and maintaining germ cell viability during larval development, while *nos-3* controlled the sperm–oocyte switch mechanism in hermaphrodites via its direct interaction with FBF, a Pumilio family protein (Kraemer et al., 1999; Subramaniam and Seydoux, 1999). Similarly, in vertebrates, *nanos* played important roles in germ cell as well. In zebrafish, *nanos1* was required for the PGCs survival during embryogenesis, and to maintain oocyte production in adult (Draper et al., 2007; Koprunner et al., 2001). Three *nanos* homologs were identified in mouse. Among them, *nanos2* and *nanos3* were involved in germ cell function. *Nanos2* knockout mice were sterile, and resulted in reduce of size and weight of testis and a complete loss of spermatogonia, while female mice appeared developmentally normal and were fertile. Characterization of *nanos3* targeted disruption revealed the totally loss of germ cells in both sexes (Tsuda et al., 2003). Later studies showed that *nanos3* was implicated in the maintenance of PGCs during migration by repression of apoptosis

Abbreviations: bp, base pairs; h, hour; UTR, untranslated region; RT-PCR, reverse transcription-polymerase chain reaction; PGCs, primordial germ cells; MS-222, 3-aminobenzoic acid ethyl ester methanesulfonate-222; SMART, switching mechanism at 5-end of RNA transcript; RACE, rapid amplification of cDNA ends; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol tetraacetic acid; PVDF, polyvinylidene fluoride; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitroblue tetrazolium; PBS, phosphate buffer solution; PI, propidium iodide.

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(Suzuki et al., 2007). As for human, there were also three *nanos* genes, and the function of *nanos2* and *nanos3* in the development of human germ cell had been particularly studied (Julaton and Reijo Pera, 2011; Kusz et al., 2009).

In addition to its germline functions, *nanos* was expressed widely in multipotent cells and somatic tissues. The homologue of *nanos* gene was identified in diverse invertebrates, such as *Nematostella vectensis* (Extavour et al., 2005), *Ilyanassa obsoleta* (Rabinowitz et al., 2008), *Hydra magnipapillata* (Mochizuki et al., 2000), *Platynereis dumerilii* (Rebscher et al., 2008), *Bombyx mori* (Zhao et al., 2008), *Hemicentrotus pulcherrimus* (Fujii et al., 2006), *Strongylocentrotus purpuratus* (Juliano et al., 2010) and so on. In sea anemone, *nanos* gene was expressed in multiple somatic types during early embryogenesis (Extavour et al., 2005), while *Hydra nanos* was expressed in multipotent interstitial cells, which produced both several somatic cell types and germ cells (Mochizuki et al., 2000). In vertebrates, such as human, *nanos1* was found in various tissues, such as brain, heart, liver, spleen, ovary, testis and so on (Julaton and Reijo Pera, 2011). By β-gal hischemical staining, mouse *nanos1* was predominantly expressed in the hippocampal formation of the adult brain, which was also observed in the cerebellum, lateral geniculate body in the thalamus, ventral tegmental area, piriform cortex, superior colliculus in the mesencephalon, and olfactory bulb (Haraguchi et al., 2003). In medaka, *nanos1* was duplicated, resulting in *nanos1a* and *nanos1b*. By in situ hybridization, *nanos1a* was detected in the nose, diencephalon, hypothalamus, caudal wall of the mesencephalon, cerebellum, and peripheral ganglia, while *nanos1b* was found in the parts of the telencephalon, nose, retina, optic tectum, mesencephalon, otic vesicle, and branchial arch; after the onset of sexual differentiation,*nanos1a* was expressed in the somatic cells surrounding the oocytes (Aoki et al., 2009). However, the expression pattern of *nanos1* in somatic tissues and its role in germ cell were still unknown.

Chinese sturgeon (*Acipense sinensis*) is one of the most primitive Actinopterygii species and belongs to Acipenseriformes, with some characters between chondrichthyes and osteichthyes. To date, it is a rare and endangered species, due to over-fishing for meat and production of caviar, destruction of their spawning grounds and other anthropogenic interferences (Birstein et al., 2002; Wei et al., 1997). To save this species more efficiently and to be able to develop an aquaculture industry, artificial propagation has been attempted since 1983. A severe problem is that Chinese sturgeon is an extremely late and asynchronous sexual maturation species. Generally, sexual maturity in males is reached between 8 and 18 years and in females between 14 and 26 years, respectively (Chen et al., 2006). However, with the improving of biotechnology, germ cell transplantation is believed to be an available and fast method for protecting this species. In order to implement this plan, germ cells must be isolated firstly. Currently, it is widely acknowledged that *nanos* can be used as a marker for germ cells (Extavour and Akam, 2003). In present study, we identified a *nanos*-related gene (*nanos1*) in Chinese sturgeon and examined its mRNA and protein expression patterns in diverse somatic tissues. Additionally, the subcellular localization of *As*Nanos1 protein in the gonad was reported.

2. Materials and methods

2.1. Animals and samples

All Chinese sturgeons used in this study were cultured in Taihu station, Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Science. Deep anesthesia was induced by a 0.05 % solution of MS-222 (Sigma, USA). The tissues samples from two and a half years old female Chinese sturgeons (about 1.11 m in length and 7.2 kg in weight) were collected within 30 min of exsanguinations by tailing and immediately dipped into liquid nitrogen and stored at −80 °C in May 2011. Additionally, surgical procedures were used to obtain the testis and ovary tissues from 4.5 years old Chinese sturgeons in May 2012. The experimental procedures were based on the standards of the Chinese Council on Animal Care.

2.2. RNA extraction and SMART cDNA synthesis

Total RNAs were extracted using SV total RNA isolation system (Promega, USA). The quality of RNAs was measured at A260 nm and the purity from the ratio A260:A280 nm (Eppendorf Biometer, Germany). Double strands cDNAs were synthesized and amplified according to the reports described previously (Li et al., 2005) using the Switching Mechanism at 5-end of RNA Transcript (SMART) cDNA Library synthesis Kit (Clontech, USA). Briefly, 100 ng of total RNAs were reverse-transcribed in 42 °C for 1 hour at the presence of both 3′ SMART CDS primer II A and SMART II oligonucleotide. And 2 μL of first-strand reaction products were used in each 100 μL long-distance PCR system containing 0.2 μM PCR primer II A. The LD-PCR parameters were 95 °C for 5 sec and 68 °C for 6 min on PTC-200 thermal cycler for 15 cycles. 5 μL of PCR products were separated and checked by electrophoresis on 1% agarose gels containing ethidium bromide.

2.3. Cloning of the full-length cDNA of Asnanos1

The full-length of *Asnanos1* cDNA was amplified by 5′- and 3′-RACE (rapid amplification of cDNA ends). Degenerate sense and antisense primers were designed and synthesized according to a nucleotide alignment of different *nanos* cDNAs. The 5′ end of the cDNA was amplified with a 5′PCR anchor primer (5′-AP, Table 1) and specific antisense primer (*Asnanos1*-5′RACE reverse). The 3′-end of *Asnanos1* cDNA was amplified using specific sense primers (*Asnanos1*-3′RACE forward1 and *Asnanos1*-3′RACE forward2) and a PCR anchor primer corresponding to the terminal anchor sequence of the cDNA (3′-AP, Table 1). All PCRs were performed on a PTC-200 thermal cycler (Bio-Rad, USA) by denaturation at 94 °C for 3 min, followed by 35 cycles of amplification at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min and an additional elongation at 72 °C for 10 min after the last cycle. The PCR mixture contained 1U Taq DNA polymerase (MBI, USA) together with 0.2 mM of each dNTPs, a suitable reaction buffer (MBI), 1.5 mM $MgCl_2$, 15 pmol of each primer and 2 μL diluted SMART cDNA. The amplified DNAs were visualized by electrophoresis of ethidium bromide stained

Table 1
Sequences of the primers used for PCR.

Primer name	Purpose	Sequence (5′ to 3′)
Degenerate_forward	Partial	TTYAAYTTYTGGAACGACTAC
Degenerate_reverse	Partial	TGRGCRTTGTCACCRTTRG
Asnanos1-3′RACE forward1	RACE	CTGCTGTTGCCCCTCTTC
Asnanos1-3′RACE forward2	RACE	TGTTACCCGATAACATCGGAGAA
Asnanos1-5′RACE reverse	RACE	ACTGCATAGGGGGCAAGTGTAA
Asnanos1-RT-F	RT-PCR	AACGACTACCTGGGACTTTCTACT
Asnanos1-RT-R	RT-PCR	ACTGCATAGGGGGCAAGTGTAA
Asβ-actin-F	Control	TGGACTTGGCTGGTCGTGAC
Asβ-actin-R	Control	CTGGCAGCTCATAGCTCTTC
Asnanos1-E-F	Express	GGATCCTTGAATGCTGGAAACTCT
Asnanos1-E-R	Express	CTCGAGTCTCTTACCCCCAACTG
3′ SMART CDS primer II A	SMART cDNA	AAGCAGTGGTATCAACGCAGAGTACT$_{(30)}$ N$_{-1}$ N
SMART II oligonucleotide	SMART cDNA	AAGCAGTGGTATCAACGCAGAGTACGCGGG
primer II A	SMART cDNA	AAGCAGTGGTATCAACGCAGAGT
3′-AP	RACE	GGTATCAACGCAGAGTACTT
5′-AP	RACE	ATCAACGCAGAGTACGCGGG

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