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Nanos2 is a molecular marker of inchoate buffalo spermatogonia

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ABSTRACT

Nanos2 belongs to the *Nanos* gene-coding family and is an important RNA-binding protein that has been shown to have essential roles in male germline stem cells development and self-renewal in mouse. However, little is known about *Nanos2* in inchoate buffalo spermatogonia. Here, rapid-amplification of cDNA ends (RACE) was used to obtain the full-length buffalo *Nanos2* sequence and bioinformatic analysis revealed a highly conserved *Nanos2* sequence between buffalo and other mammalian species. Although *Nanos2* was expressed in various tissues, the highest mRNA expression levels were found in testes tissue. Moreover, *Nanos2* mRNA was abundant in fetal and pre-puberal testes but markedly decreased in the testes of adults. At the protein level, immunohistochemistry in pre-puberal testes revealed a pattern of NANOS2 expression similar to that for the undifferentiated type A spermatogonia marker PGP9.5. Furthermore, NANOS2 expression was low in adult testes and restricted to elongating spermatids. Altogether, our data suggest that *Nanos2* is a potential preliminary molecular marker of inchoate buffalo spermatogonia, and may play an important role in buffalo spermatogonial stem cells (SSCs) development and self-renewal, as has been observed in other model animals.

1. Introduction

Spermatogonial stem cells (SSCs) are a type of adult stem cell that resides in the testes. They are able to maintain their self-renewal ability, and can differentiate through spermatogenesis to generate the male gamete (Rooij and Russell, 2000). Given their important role in male reproduction and their potential applications in assisted reproduction and male fertility preservation, it is crucial to develop strategies to identify and isolate SSCs from the adult testes. In the mature testes, SSCs reside adjacent to the basement membrane of the seminiferous tubules in a privileged niche supported by surrounding Sertoli cells (Roosen-Runge and Giesel, 1950; Rooij et al., 2008). However, considering that in the adult testes SSCs represent a very small proportion of all the germ cells in the adult testes (~0.03% in the mouse) (Tagelenbosch and Rooij, 1993), we usually use some immunocytochemical markers to identify their histological location.

Abbreviations: SSCs, spermatogonial stem cells; PGCs, primordial germ cells; RACE, rapid-amplification of cDNA ends; *Gfra1*, glial cell derived neurotrophic factor receptor alpha 1; *Oct-4*, POU domain class 5 transcription factor 1 (POU5F1); *Nanos2*, nanos C2HC-type zinc finger 2; *Plzf*, promyelocytic leukemia zinc finger; *Thy-1*, thymus cell antigen 1; *Etv-5*, ets variant 5; *Ddx4*, DEAD (Asp-Glu-Ala-Asp) box polypeptide 4; *Gata4*, GATA binding protein 4; *Stra8*, stimulated by retinoic acid gene 8; *Scp3*, synaptonemal complex protein 3

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Indeed, in several species, SSCs can be identified on the basis of the expression of a combination of biomarkers (Martin and Seandel, 2013). For instance, mouse SSCs express surface markers such as glial cell derived neurotrophic factor receptor alpha 1 (*GFRα1*) (Buageaw et al., 2001), cadherin-1 (*CDH1*)(Tokuda et al., 2007) and thymus cell antigen 1 (THY1, also known as CD90) (Shinohara et al., 2000; Kubota et al., 2004a, 2004b; Reding et al., 2010). Intracellular markers for mouse SSCs may include promyelocytic leukemia zinc finger (PLZF, also known as ZBTB16) (Costoya et al., 2004) and, POU domain class 5 transcription factor 1 (POU5F1, also known as OCT 4) (Dym et al., 2009) among others. However, there are differences among species regarding the combination of specific SSC biomarkers, and these have been less well characterized in domestic and livestock species; thus more specific markers are needed in buffalo research. For instance, ubiquitin C-terminal hydrolase-L1 (*UCH-L1*, previously known as protein gen product 9.5 (*PGP9.5*)) has also been defined as a marker for bull (Wrobel 2000), pig (Luo et al., 2006) and sheep (Rodriguez-Sosa et al., 2006) SSCs, and PLZF as an intracellular marker for ovine (Borjigin et al., 2010) and horse (Costa et al., 2011) SSCs. Notably, the application of a combination of both positive and negative markers was required to isolate an enriched population of SSCs (Shinohara et al., 2000; Kubota et al., 2004a, 2004b).

Buffalo (*Bubalus bubalis*) is an economically important livestock source in southwest China, but their reproductive efficiency is very low because of delayed puberty, seasonality, anestrus, low conception rates, and long calving intervals (Perera 2008). Little is known about buffalo SSCs, and their characterization may provide avenues for understanding and improving male fertility in this species. Therefore, the study of buffalo SSCs has great significance for buffalo breeding improvement, as well as for contributing to our knowledge of basic developmental biology. The *Nanos* gene encodes for an RNA-binding protein that was first identified in *Drosophila* (Wang and Lehmann, 1991) and has been proposed as a conserved factor for germline stem cell function. In *Drosophila*, primordial germ cells (PGCs) fail to migrate into the gonad and do not become functional germ cells if maternal *Nanos* is absent (Wang and Lehmann, 1991; Kobayashi et al., 1996). In mouse, NANOS2 has been reported to be a specific marker of male germinal stem cells, and is required to maintain SSCs (Tsuda et al., 2003; Suzuki et al., 2007; Sada et al., 2009). Therefore, the objective of this study was to characterize the expression of *Nanos* in buffalo male reproductive tissue and germ cells, as a potential marker for SSCs in this species. We used rapid-amplification of cDNA ends (RACE) to obtain the full-length sequence of buffalo *Nanos2* gene, and used bioinformatics to predict the corresponding aminoacid sequence. We then compared mRNA and protein expression levels in fetal, pre-puberal and adult buffalo testicular tissue. These results represent an advance in the characterization of SSCs in buffalo as a potential tool for fertility preservation in this species.

2. Materials and methods

2.1. Ethics statement

All animal procedures used in this study were carried out in accordance with the Guide for Care and Use of Laboratory Animals (8th edition, released by the National Research Council, USA) and were approved by the Institutional Animal Care and Use Committee (IACUC) of Guangxi University.

2.2. Animals and tissue collection

The testes from fetal (about 2–4 months after pregnancy, n = 3), pre-puberal (about 4–6 months old, n = 3) and adult (about 2–3 years old, n = 3) buffalo were collected from a local abattoir (Nanning, China). Within 2 h after death, the pre-puberal and adult testes were transported to the laboratory in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco, CA, USA) containing 5 × Penicillin/Streptomycin (Gibco) on ice. The whole fetus was transported to the laboratory in saline solution at 37 °C. Various fetal tissues including testes, brain, heart, ear, liver, spleen, lung and kidney were collected. Total RNA was extracted from these tissues using the Total RNA Kit I (OMEGA, GA, USA). RNA concentrations were quantified using EPOCH™ (BioTek, VT, USA). Total RNA was stored at −80 °C for later use.

2.3. Rapid amplification of cDNA ends (RACE)

Total RNA of fetal buffalo testes were used to generate full length *Nanos2*. Rapid amplification of cDNA Ends (5′- and 3′- RACE) was performed with the SMARTer® RACE 5′/3′ Kit (TaKaRa, NO.634860, Dalian, China), following the manufacturer's instructions. The gene specific primers were designed using Primer Premier (Ver 6.0) (Canada). Since according to the kit we chose in-fusion cloning and touch-down PCR, gene specific primers were designed according to the sequence in the database as follows: Forward primer:5′-GATTACGCCAAGCTTCAGTCTCTCTACCGCCGCAGTGG-3′; Reverse primer:5′-GATTACGCCAAGCTTTGGCCCCGCACAGGGGACATA-3′. The purified PCR products were cloned into the in-fusion vector provided by the SMARTer® RACE 5′/3′ Kit (TaKaRa, NO.639648, Dalian, China) prior to sequencing by IGEbio (Guangzhou, China).

2.4. Bioinformatic analysis

Using the RACE, we obtained the full fragment of the buffalo *Nanos2* gene, which was then successfully sequenced by IGEbio. The homology of the *Nanos2* gene sequence between buffalo and other mammal species was analyzed with NCBI BLAST tools (Nucleotide BLAST). Phylogenetic trees were constructed using MEGA7.0.18 software (PA, USA). Conserved domains were identified by NCBI CD-search (CD-search) and NCBI protein BLAST (Protein BLAST).