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ORIGINAL RESEARCH ARTICLE

Cloning and Characterization of a *Nanog* Pseudogene in Sika Deer (*Cervus nippon*)

Datao Wang,[1,2] Qianqian Guo,[1] Hengxing Ba,[1] and Chunyi Li[1,2]

Nanog plays a crucial role in the maintenance of stem cell pluripotency. Annual full regeneration of deer antlers has been shown to be a stem cell-based process, and antler stem cells (ASCs) reportedly express *Nanog*. In the present study, we found that *Nanog* RNA expressed by ASCs was a pseudogene (*Nanog-ps*). The coding sequence of *Nanog-ps* was 93.1% homologous to that of bovine *Nanog*, but with two missing nucleotides after position 391. Deletion of the two nucleotides in *Nanog-ps* resulted in a frame-shift mutation, suggesting that *Nanog-ps* would not encode a normal Nanog protein. Overexpression of *Nanog-ps* failed to affect downstream genes of *Nanog* or to enhance cell proliferation in the ASCs. However, this pseudogene was transcribed in the ASCs and encoded a nuclear protein; the expression levels of *Nanog-ps* were also related to the degree of stemness in antler cells. Here, we reported this pseudogene, because it could serve as a useful marker for identifying ASCs and evaluating the degree of their stemness.

Introduction

Oct4, Sox2, and Nanog are the key regulators for self-renewal and pluripotency in embryonic stem cells (ESCs) (Niwa *et al.*, 2000; Avilion *et al.*, 2003). Oct4 and Sox2 are a part of the core network of transcription factors supporting pluripotent cell self-renewal, and Nanog interacts with Oct4 and Sox2 to maintain pluripotency (Boyer *et al.*, 2005). Somatic cells can be reprogrammed into pluripotent cells by inducing the expression of these transcription factors (Takahashi and Yamanaka, 2006; Yu *et al.*, 2007). In transcription factor-induced reprogramming, Nanog is initially dispensable but in the final stages of reprogramming its role has been found to be essential. Nanog may, however, be induced by the preceding factors, and its endogenous expression may be sufficient for pluripotent reprogramming. Based on these studies, Silva *et al.* (2009) claimed that Nanog acts as a gateway to the pluripotent state of ESCs.

Deer antlers are the only mammalian appendages that are capable of full renewal, and both antler development and regeneration are stem cell-based processes (Li *et al.*, 2009). The antler stem cells (ASCs) are reported to express several key genes that are associated with ESCs (such as *Oct4*, *Sox2*, and *Nanog*), and they can be induced to differentiate into multiple cell lineages (Li and Suttie, 2001). However, thus far, no study was pursued along this discovery. Hence, in the present study, we investigated the expression of *Nanog* in the ASCs.

Previous studies reported that *Nanog* pseudogenes exist in several species of mammals. For example, four *Nanog* pseudogenes are found in the mouse genome (Saunders *et al.*, 2013); two are found in the porcine genome (Yang *et al.*, 2014); and 10 pseudogenes and one tandem duplicate are found in the human genome (Booth and Holland, 2004). The term *pseudogenes* is used to describe sequences that are found to be both related and defective to functional genes (Vanin, 1985). Long *et al.* (1999) stated that the pseudogenes may lead to the formation of new gene products. As such, some pseudogenes may not be completely inactive; the human *Nanog* pseudogene *NanogP8* has been found to express functional protein in both smooth muscle cells and prostate cancer cells, and it was able to promote cell proliferation (Jeter *et al.*, 2009).

In the present study, we demonstrated and characterized a pseudogene of *Nanog* (*Nanog-ps*) in sika deer. This raises the possibility that the *Nanog* expressed in the ASCs previously reported (Li *et al.*, 2009) may only be a pseudogene. *Nanog-ps* has no known functions; however, it has preserved some attributes of real *Nanog*, such as nuclear location and its relationship with antler cell stemness. Here, we reported this pseudogene, because it would still serve as a useful marker for identifying ASCs and evaluating the degree of their stemness, even if it is demonstrated to be functionless in future studies.

Materials and Methods

Animals and cell types

Blood samples of 11 deer (four sika deer, three red deer, two muntjacs, and two reindeer), two bovines, and two

[1]Chinese Academy of Agricultural Sciences, Institute of Special Wild Economic Animal and Plant Science, Jilin, China.
[2]State Key Laboratory for Molecular Biology of Special Economical Animals, Chinese Academy of Agricultural Sciences, Jilin, China.

Table 1. Sequence of Primers Used in This Study

Primers no.	*Primers sequences (5′-3′)*	*Product size (bp)*	*Target gene*
1	FS: CTTCAGCCTGGACTTTTCCTA RS: GGTACTACTTATGATGTGGCAA	1461	*Nanog* cDNA*/Nanog-ps*
2	FS: CACCCTCGACACGGACACT RS: CTGCTTGTAGCTGAGGTTCAA	2148/281	*Nanog* DNA /*Nanog-ps*
3	FS: GACAGTCCTGATTCTTCCACAA RS: CCATTTCTTACATTTCATTCTCTG	1467/268	*Nanog* DNA /*Nanog-ps*
4	FS: GACTCAGCACTGCCAATGAT RS: GACCTCCGATATTCCAATGCA	1202	*Nanog* DNA
5	FS: GAAATGTAAGAAATGGCAGAAAAAC RS: CAAAATACAAAACATCGTTCTCCA	1118	*Nanog* DNA
6	FS: CAGTTGTGTGTGCTCAATGAC RS: TCCAGGAGAGTTCACCAAAC	1801/263	*Nanog* DNA/cDNA
7	FS: GTTGTGTGTGCTCAATGAGAT RS: TCCAGGAGAGTTCACCAAAAC	260	*Nanog-ps*
8	FS: CTTAAGCTTTTTCCCATCTTCTTCA RS: TAGTCGACAATAGCCGTCACAAAT	963	*Nanog-ps*
9	FS: CTTAAGATGAGTGTGGATCCAGCTTG RS:GTCGACGTTTCACTCATCTTCACACGT	895	*Nanog*
10	FS: CCAGCCTTGGAACAATCAGT RS: GTTTGGGAATAAATCCGTGAAT	186	*Nanog-ps*
11	FS: CTCTGACGGTCTGGGAGGAG RS: GGGCAGCAACAAGGAATAAA	243	*MMP3*
12	FS: GCTGCGACTCACATCATAAGGG RS: GAGATGCATGTGGTCCAGGAT	194	*Wnt3*
13	FS: TTTTACAGAACGGGAGGATAAGC RS: GAGTCGCATCCACCCACCTA	174	*TGFβ1*
14	FS: CCGAGACATCAAGGAGAAGCTG RS: GTAGTTTCGTGAATGCCGCA	206	β-*actin*

FS, forward strand; RS, reverse strand.

sheep aged from 2 to 5 years old were collected from the jugular vein into 5 mL anticoagulative tubes. The ASCs, including antlerogenic periosteal cells (APCs), pedicle periosteal cells (PPCs), and antler tip reserve mesenchymal cells (ATRMCs), were obtained and cultured according to the previous methodology (Li *et al.*, 2002). Facial periosteal cells (FPCs) served as a control (Li *et al.*, 2012). Each cell type was cultured in three independent biological replicates. Blood sampling from living animals and tissue collection from the slaughtered deer heads were approved by the Ethical Committee of the Chinese Academy of Agricultural Sciences for the use of Animals (CAAS201357). All cell types were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum, 100 mg/mL of streptomycin, and 100 U/mL of penicillin, known as complete medium, and grown in a humidified atmosphere with 5% CO_2 at 37°C.

Preparation of DNA and RNA, PCR and qPCR

Genomic DNA was isolated from the collected blood samples using a Genomic DNA purification kit (Qiagen, Dusseldorf, Germany). Total RNA was extracted from the APCs, the PPCs, the FPCs, and the ATRMCs using a Trizol total RNA isolation kit (Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg of total RNA (DNase-treated) using a Primescript RT-PCR kit (Takara, Dalian, China).

Polymerase chain reaction (PCR) amplifications of genomic DNA and cDNA were performed in 50 μL solution containing 1 × buffer, 0.25 mM of each dNTP, 1 mM of each primer, and 1 U of Ex Taq (Takara). PCR reaction conditions were as follows: 2 min at 94°C, followed by 30 amplification cycles (94°C for 30 s, 30 s at specific primer annealing temperature, and 72°C for 90 s). The size and purity of the PCR products were estimated by subjecting samples (5 μL) to 1% agarose gel electrophoresis. The purified PCR products were directly sequenced by Sangon (Shanghai, China). *Nanog* of the sika deer was amplified from the genomic DNA and sequenced using splicing overlap extension. Quantitative polymerase chain reaction (qPCR) was performed on ABI 7500 (Applied Biosystem, Singapore, Singapore) using SYBR Green (Qiagen) according to the manufacturer's protocol. The relative expression level of genes was analyzed by the $2^{-\Delta\Delta}$Ct method by using β-*actin* for normalization. All primers used in this study are given in Table 1.

Chondrogenic differentiation

The APCs were induced to chondrogenic differentiation at passage 3 or 4 using a previously described micromass culture (Sun *et al.*, 2012). Briefly, the APCs were resuspended with chondrogenic medium (DMEM +10 ng/mL TGFβ1 + 0.1 μM dexamethasone +50 mg/mL ascorbate-2-phosphate) to a concentration at 1×10^8 cells/mL, and they were seeded in six-well plates with 100 μL/well. The plate

was incubated for 4 h at 37°C before 2 mL chondrogenic medium was added. The chondrogenic medium was changed every 2 days. The resultant pellets were harvested 3 weeks after initial seeding. For qPCR analysis, RNA was extracted from pellets as mentioned earlier. For histology, each pellet was fixed in 10% buffered formalin followed by paraffin embedding. The pellet sections (5 μm thick) were stained with hematoxylin/eosin for general morphology, and with hematoxylin/alcian blue for the detection of cartilage matrix.

Plasmid construction and gene transfection

The retroviral vector pLEGFP-C1 (Clontech, San Francisco, CA) was used to express Nanog-ps and Human Nanog. T-vector containing human *Nanog* was gifted by the Stem Cell Research Center of Jilin University. The *Nanog-ps* and *Nanog* were amplified with primers containing *Hin*dIII/*Sal*I sites (Primer 8 and 9). Purified fragments (using DNA Pure Kit; Takara) and pLEGFP-C1 vector were digested with *Hin*dIII and *Sal*I (Promega, Madison, WI) and ligated with T4 DNA Ligase (Promega). Recombinant vectors were transfected into the ASCs using Lipofectamine 2000 reagent (Qiagen) according to the manufacturer's protocol. Transfected cells were selected for neomycin resistance in the complete medium containing 150 μg/mL G418 (Sigma, St. Louis, MO). Transfection efficiency of 30,000 cells was analyzed by FACSCalibur flow cytometry (BD Biosciences, San Diego, CA).

MTT assay

The MTT assay was conducted to evaluate ASCs cell proliferation in 96-well plates, using the methodology described elsewhere (Maioli *et al.*, 2009). Briefly, 200 μL complete medium containing 5000 cells was added to each well, and each sample was tested in triplicate. Cells were incubated for predetermined time periods (0, 24, 48, 72, and 96 h) in a 37°C incubator that was supplemented with 5% CO_2. After incubation, 20 μL MTT reagent (5 mg/mL) (Sigma) was added to each well and incubated for 2 h until the purple precipitate was visible; the medium was then carefully removed, and 150 μL DMSO was added. Plates were shaken in the dark for 10 min, and the OD value was read at 490 nm using an ELISA reader (TECAN, Grodig, Austria).

Cell cycle analysis

Cell cycles were analyzed using flow cytometry after staining with propidium iodide (PI) (Sigma), using the method reported elsewhere (Pozarowski and Darzynkiewicz, 2004). Briefly, 1×10^6 cells were seeded in each T75 flask, cultured for 48 h, digested with trypsin, and washed with precooled PBS. Next, the cells were resuspended and fixed in 75% alcohol overnight. Fixed cells were centrifuged at 300 *g* for 5 min and washed twice with PBS. The cells were then resuspended in 500 μL PBS containing 100 μg/mL RNase (Sigma), and they were incubated at 37°C for 30 min. The mixture was filtered through a 200 μM mesh sieve, and PI was added to the filtrate to a final concentration of 10 μg/mL; samples were kept in the dark at 4°C for 10 min. Thirty thousand cells of each sample were analyzed using FACSCalibur flow cytometry (BD Biosciences), and the data were processed with MultiSET software (BD Biosciences).

Statistical analysis

Data were presented as the mean ± SEM in three or more independent experiments. Statistical analysis was performed with *T*-test using SAS (Statistical Analysis System) version 9.0, and values at $p < 0.05$ were considered significant.

Results

Cloning and characterization of Nanog-ps

In the present study, the coding DNA sequence (CDS) of *Nanog* was cloned from the ASCs of sika deer using reverse transcription-polymerase chain reaction (RT-PCR) (Primer 1; Table 1) and sequenced in both directions. The CDS was then compared with that of *Nanog* from other mammalian species: *Bos taurus* (GI: 70778751), *Sus scrofa* (GI: 749320089), *Homo sapiens* (GI: 663071049), and *Mus musculus* (GI: 577019512). The results showed that the CDS of sika deer was highly similar to that of *Bos Taurus* (93.1%); however, there was a two-nucleotide frame shift after nucleotide position 391 (Fig. 1A). The sequence has been submitted to GenBank (KR005602). To analyze the predicted protein encoded by this mutant gene, the sequence was analyzed by blast of SwissProt, and a premature termination codon was detected due to the frame shift, which would result in a truncated polypeptide containing 144 amino acids (Human Nanog contains 305 amino acids, and bovine contains 300 amino acids) (Fig. 1A). Therefore, in this article, the sequence was named *Nanog-ps*.

To further investigate *Nanog-ps*, genomic *Nanog* from sika deer was cloned (Primer 1–5; submitted to GenBank: KR559685), and no gap was detected in genomic *Nanog* (Fig. 1B). In the process of amplifying sika deer genomic DNA, we detected one shorter *Nanog* homologous sequence (Primer 3) (Fig. 1C) and this sequence was identical to *Nanog-ps*. We amplified and sequenced genomic *Nanog-ps* (Primer 1), and we found that *Nanog-ps* was an intronless pseudogene. *Nanog* of sika deer was composed of four exons and three introns (Fig. 1D); sequencing results in the present study showed that *Nanog-ps* shared 98% homology with *Nanog* CDS of sika deer.

Nanog-ps*, a deer family-specific gene*

To determine whether the *Nanog-ps* was deer family specific, three other deer species, red deer (*Cervus elaphus*), muntjak (*Muntiacus muntjak*), and reindeer (*Rangifer tarandus*) were selected and their genomic DNA was analyzed using the previously designed primers (Primer 2). The results showed that these four deer species had the same electrophoretogram (Fig. 2), whereas no *Nanog-ps* or similar sequence was found in *Bos taurus* and *Ovis aries*. *Nanog-ps* of each deer species was sequenced and submitted to NCBI (KR005602–KR005605).

Nanog *mRNA was absent in the ASCs*

Previous studies showed the existence of *Nanog* mRNA in the ASCs (Li *et al.*, 2009). In this study, we found *Nanog-ps* expressed in the ASCs, and the coding region of *Nanog* was 98% homologous to that of *Nanog-ps*. To discriminate

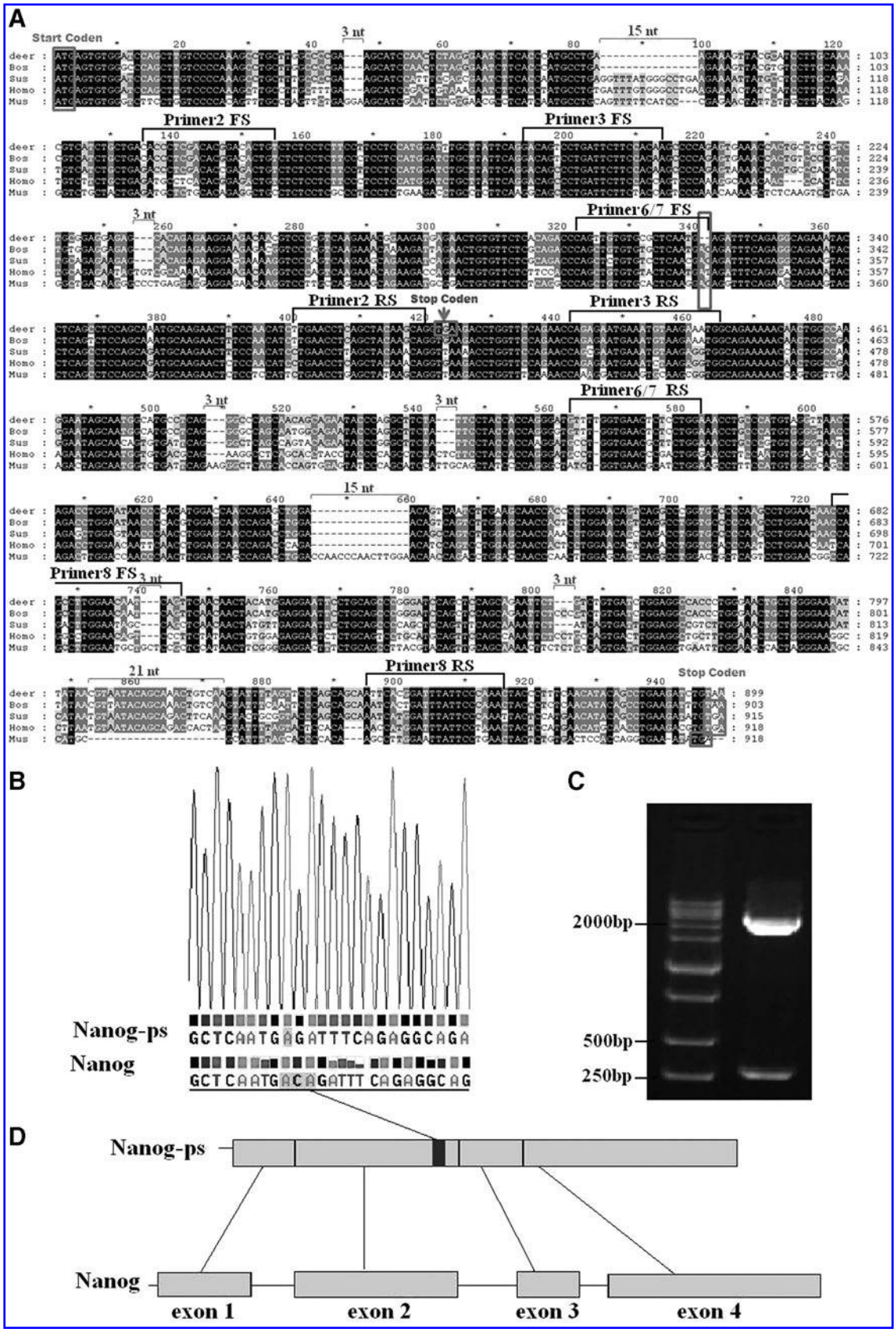

FIG. 1. Characterization of *Nanog-ps*. **(A)** Sequence alignment between *Nanog-ps* and *Nanog* coding sequences from *Bos taurus*, *Ovis aries*, *Homo sapiens*, and *Mus musculus*. Note the two nucleotides missing (in the *red box*). **(B)** Alignment of the DNA fragments of *Nanog-ps* and *Nanog* containing the site of deletion. **(C)** Two bands were detected in sika deer genomic DNA: a large band (*Nanog*) and a small band (*Naong-ps*). **(D)** Gene structures of *Nanog* and *Nanog-ps* in sika deer: The former was composed of four exons with three intervening introns, and the latter was an intronless pseudogene.

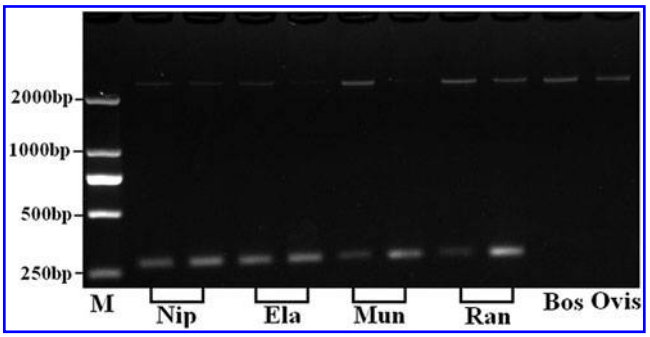

FIG. 2. Detection of *Nanog-ps* in the selected four deer: one Ovis and one bovine species through PCR. Four deer species showed an identical electrophoretogram, and each had two bands with a different size: The larger one was a part of *Nanog* containing an intron, and the smaller one was a part of *Nanog-ps*. Nip, *Cervus nippon*; Ela, *Cervus elaphus*; Mun, *Muntiacus muntjak*; Ran, *Rangifer tarandus*; Bos, *Bos Taurus*; Ovis, *Ovis arise*; PCR, polymerase chain reaction.

between *Nanog* mRNA and *Nanog-ps* mRNA, we designed two pairs of primers at the mutation position (Primers 6/7). The results of the RT-PCR showed that *Nanog* mRNA was undetectable in the ASCs and the FPCs (Fig. 3A), whereas *Nanog-ps* mRNA was found in these cell types examined (Fig. 3B).

Expression and localization of Nanog-ps

To analyze the expression patterns of *Nanog-ps* in the ASCs, we examined the levels of mRNA expression in the ASCs (APCs, PPCs, and ATRMCs), the FPCs, and the chondrocytes derived from the APCs. The results of our qPCR showed that the expression levels of *Nanog-ps* in the APCs were significantly higher than those in the PPCs; the PPCs were significantly higher than the ATRMCs; and the ATRMCs was significantly higher than the FPCs and the chondrocyte pellets derived from APCs ($p < 0.05$) (Fig. 4A). Histological examination showed that the APCs formed chondrocyte-like cells and matrix in the micromass-cultured pellets (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/dna). To determine the subcellular localization of Nanog-ps, the APCs were transfected with recombinant vectors pLEGFP-Nanog-ps, and with pLEGFP-C1 (mock vehicle) as a control. The results showed that Nanog-ps-GFP was located in the nucleus, whereas GFP was distributed in whole cells (Fig. 4B).

Functional analysis of Nanog-ps

It has been reported that Nanog regulates cell cycle progression and cell proliferation of stem cells (Zhang *et al.*, 2005). To determine whether Nanog-ps has this potential function, the APCs and the ATRMCs were transfected with pLEGFP-Nanog-ps and pLEGFP-Nanog. In the flow cytometry analysis, the percentages of fluorescent cells were very high (more than 81%) (Supplementary Fig. S2). The results of MTT assay showed that expression of *Nanog* significantly enhanced proliferation of the APCs and the ATRMCs, whereas there were no significant differences in cell proliferation detected between the cells transfected with plEGFP-Nanog-ps and with mock vehicle (Fig. 5A). Expression of *Nanog* promoted the APCs and the ATRMCs to enter into S-phase, whereas up-regulation of *Nanog-ps* failed to change cell cycle distribution (Fig. 5B). To further analyze the effects of overexpression of *Nanog-ps*, downstream genes of *Nanog*, *TGFβ1*, *MMP3*, and *Wnt3*, which were reported by previous studies (Liu *et al.*, 2009; Han *et al.*, 2012), were detected by qPCR in this study. The results showed that these downstream genes of *Nanog* did not respond to overexpression of *Nanog-ps* (Fig. 5C).

Discussion

In the present study, we isolated and characterized the *Nanog-ps*, a Nanog-like coding sequence, in sika deer. The sequence of the *Nanog-ps* was 98% homologous to the coding region of *Nanog*. Deletion of two nucleotides in the *Nanog-ps* resulted in a reading frame shift mutation, suggesting that the *Nanog-ps* would not encode a normal Nanog protein (Fig. 1). However, this pseudogene retained the ability to be transcribed in the ASCs. We further demonstrated that the ASCs expressed the *Nanog-ps* rather than *Nanog*, and *Nanog-ps* encoded a nuclear protein; additionally, we found that the expression level of *Nanog-ps* was related to the degree of stemness of ASCs.

Not only sika deer but also three other deer species expressed *Nanog-ps* (Fig. 2); whereas *Bos taurus* and *Ovis aries*, which have a close genetic relationship to the deer

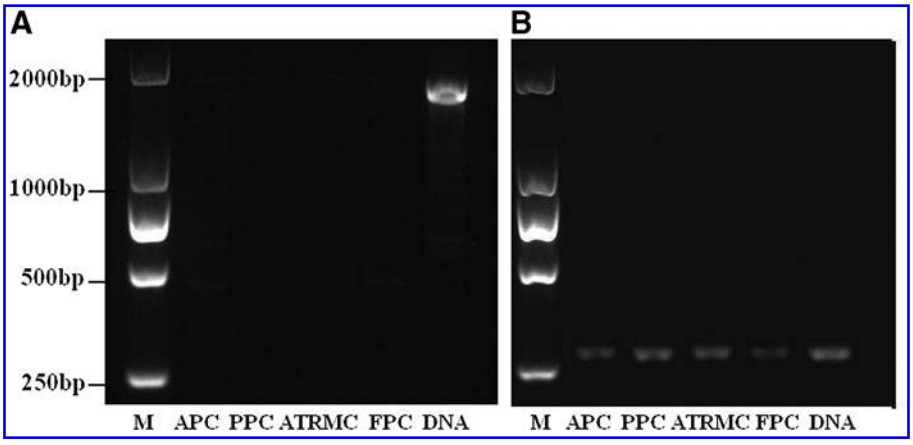

FIG. 3. Detection of *Nanog-ps* in the selected four types of deer cell mRNA and one DNA sample using RT-PCR. **(A)** *Nanog* mRNA was undetectable in the APCs, the PPCs, the ATRMCs, and the FPCs. **(B)** *Nanog-ps* mRNA was present in the APCs, the PPCs, the ATRMCs, and the FPCs. Genomic DNA of sika deer served as the positive control. APC, antlerogenic periosteal cell; ATRMC, antler tip reserve mesenchymal cells; FPC, facial periosteal cell; PPC, pedicle periosteal cells; RT-PCR, reverse transcription-polymerase chain reaction.

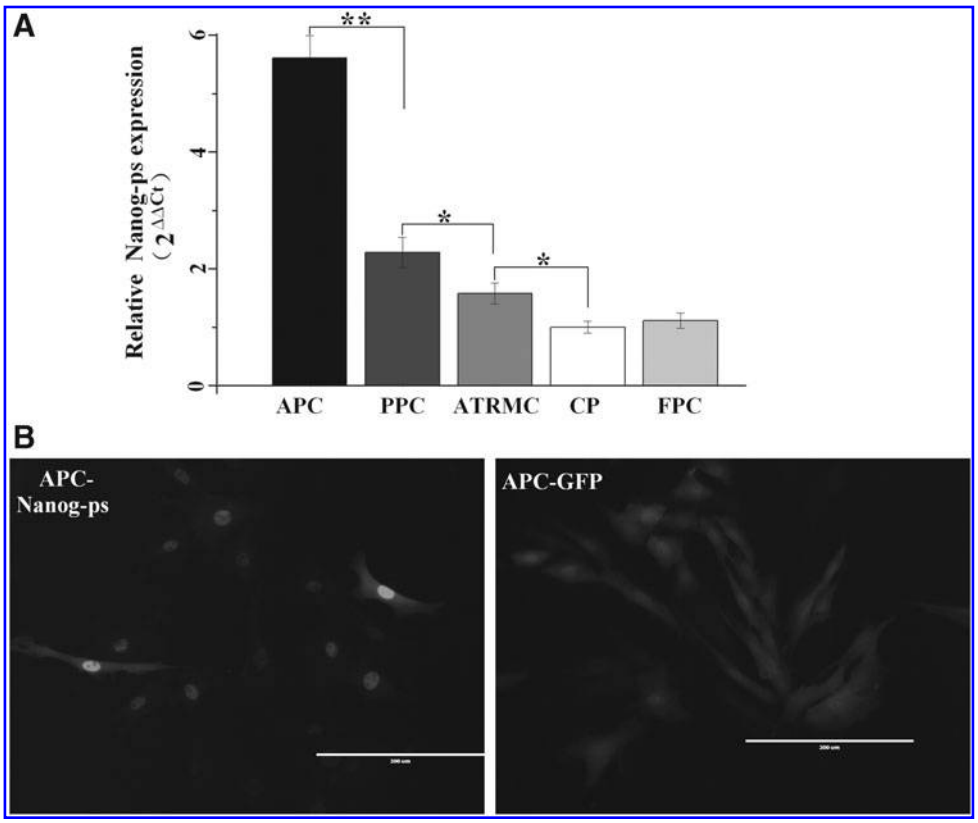

FIG. 4. Differential expression and intracellular localization of Nanog-ps. **(A)** Expression levels of *Nanog-ps* in different deer cell types through qPCR. Note that the expression levels showed a high-to-low transition in the ASCs lineages (APCs to the ATRMCs), and they were significantly higher in the ASCs than that in the FPCs and chondrocyte pellets ($p < 0.05$). Values are the mean of three separate experiments in quadruplicates and are expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$. **(B)** Intracellular localization of *Nanog-ps* in the APCs under a fluorescent microscope. Note that the expression of *Nanog-ps* was only localized in the nucleus of APCs. ASC, antler stem cell; CP, chondrocytes pellets; SD, standard deviation.

family, did not. It is reported that the age of pseudogenes can be estimated by counting the total number of mutations that had occurred in the corresponding region (Nachman, 2004). Based on the calculated figure on the rate of mutation (Booth and Holland, 2004), 1.25×10^{-9} mutations per site per year, including the contributions of insertions and deletions, we encountered 25 mutations across 900 nucleotides, with the estimated age of the *Nanog-ps* being around 22 million years, which coincides with the time of appearance of the deer family during evolution (about 20 million years ago) according to fossil records (Slate *et al.*, 2002). Given that the *Nanog-ps* was only detected in the selected deer species, and not in the selected bovine or ovine species, and the time of the appearance of *Nanog-ps* coincided with that of the deer family, we tentatively conclude that *Nanog-ps* was a pseudogene that is specific to the deer family.

The *Nanog-ps* was not a completely inactive gene. *Nanog-ps* maintained the ability to transcribe in the ASCs. Our results further showed that the mRNA expression levels of *Nanog-ps* were different in different deer cell types (Fig. 4A). Based on the previous studies, the PPCs are derived from the APCs during development of the deer pedicles, and the ATRMCs are derived from the PPCs during generation and regeneration of deer antlers (Li *et al.*, 2009). In this study, chondrocytes were derived from the APCs. Rolf *et al.* (2008) reported that both ATRMCs and PPCs were able to differentiate along the osteogenic lineages. Our study showed that the expression level of *Nanog-ps* matches the differentiation degree of the ASCs during antler development, which is consistent with the report that *Nanog* mRNA is down-regulated during mouse embryo development (Chambers *et al.*, 2003).

Nanog is a transcriptional factor that is located in the cell nucleus. It is reported that human Nanog has a nuclear localization signal in the homeodomain that is required for nuclear localization (Do *et al.*, 2007). Nanog-ps was also found to be located in the nucleus of the ASCs in our study (Fig. 4B). As one of the key transcriptional factors involved in the maintenance of self-renewal and pluripotency of ESCs, Nanog would be expected to be localized to the nucleus, where it would regulate transcription (Chang *et al.*, 2009). Nanog-ps retained this feature of real Nanog. It is reported that Nanog regulates the cell cycle process and cell proliferation of ESCs (Zhang *et al.*, 2005); to verify whether Nanog-ps has similar functions, the APCs and the ATMRCs were transfected with Nanog-ps overexpression vector. Overexpression of *Nanog-ps* could not, however, enhance cell proliferation rates or change cell cycle distributions (Fig. 5). Furthermore, downstream genes of *Nanog* did not respond to the overexpression of *Nanog-ps*. Due to the frame shift, it is expected that *Nanog-ps* may not exert a role similar to that of real *Nanog*.

We wonder whether *Nanog-ps* is an inactive gene or a totally new gene. The term *pseudogenes* is used to describe sequences that are similar to normal genes and are regarded as defunct relatives of functional genes (Vanin, 1985). However, some reports have suggested that some pseudogenes may not be completely inactive sequences, and in some cases, the presumed pseudogenes are actually new duplicated genes with new functions (Balakirev and Ayala, 2003; Harrison *et al.*, 2005). *Nanog-ps* may be a production

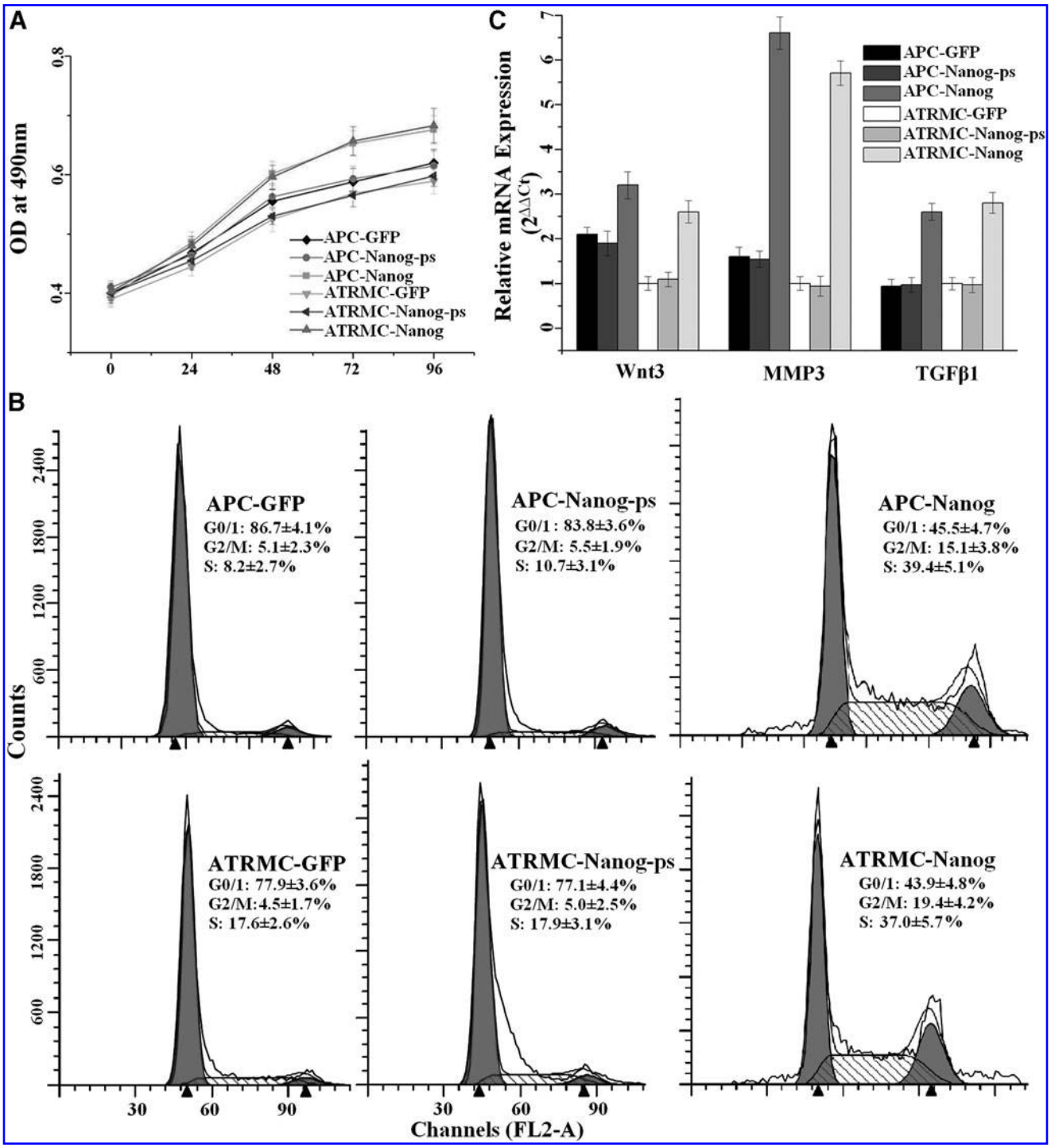

FIG. 5. Effects of overexpression of *Nanog-ps* on proliferation, cell cycle, and expression of three *Nanog* downstream genes in the APCs and the ATRMCs. **(A)** Cell proliferation profiles of the APC-GFP, the AP-Nanog-ps, the APC-Nanog, the ATRMC-GFP and the ATRMC-Nanog-ps, and the ATRMC-Nanog. **(B)** Cell cycle distributions of the APC-GFP, the AP-Nanog-ps, the APC-Nanog, the ATRMC-GFP, the ATRMC-Nanog-ps, and the APC-Nanog. **(C)** Expression of *Nanog* downstream genes (*Wnt3*, *MMP3*, and *TGFβ1*) through qPCR in the ASCs. Note that overexpression of *Nanog-ps* did not increase the proliferation rate, did not change the cell cycle distribution, and did not alter the expression levels of the *Nanog* downstream genes in the ASCs; whereas overexpression of *Nanog* (positive control) significantly increased the proliferation rate, changed the cell cycle distribution, and altered the expression levels of the *Nanog* downstream genes either significantly ($p < 0.05$) or highly significantly ($p < 0.01$).

of retrotransposition. Retrotransposition refers to the insertion of DNA sequences that are reverse transcribed from an mRNA intermediate; pseudogenes are, therefore, characterized by the absence of introns. Retrotranspositional genes are an important source of material for new gene formation during evolution (Ewing *et al.*, 2013). *Nanog-ps* showed a positive correlation with stemness and the feature of nuclear localization; hence, we cannot rule out the possibility that *Nanog-ps* is a new candidate gene, although no function was demonstrated in the present study.

Even if it is confirmed to be functionless in future studies, *Nanog-ps* may still serve as a useful marker for the ASCs. Nanog is highly expressed in ESCs and down-regulated on differentiation (Choi *et al.*, 2012), whereas we found for the first time that *Nanog-ps,* a pseudogene of *Nanog*, was down-regulated on cellular differentiation in the ASCs, that is, *Nanog-ps* was related to the degree of stemness of antler cells. A reliable cell marker for the ASCs would undoubtedly help us in identifying and evaluating antler cell stemness.

In the present study, we demonstrated that the ASCs expressed Nanog-ps rather than Nanog (Fig. 3). This finding has raised concerns as to how the APCs retain their multipotency without Nanog. Although the expression of Nanog is a gateway to the pluripotent state of ESCs (Silva *et al.*, 2009), the role of Nanog in adult stem cells (such as MSCs) is, thus far, not clear. For example, some studies show that MSCs express Oct4 or Nanog (Pochampally *et al.*, 2004; Greco *et al.*, 2007); Liedtke *et al.* (2007) indicated that many detected signals of pluripotency genes may come from their pseudogenes. Overexpression of Oct4 or Nanog would enhance the potential of proliferation and differentiation of these cells (Tsai *et al.*, 2012); however, knockout of either of the genes did not affect the ability of these cells to form colonies, and to differentiate into bone, fat, or cartilage (Lengner *et al.*, 2007). Thus, the presence and the roles of pluripotency genes in adult stem cells still remain to be elucidated. Another point for the reason that adult stem cells may not necessarily express Nanog is that, in definition, adult stem cells only possess multipotency rather than pluripotency (Kuci *et al.*, 2009).

In our previous study (Li, 2012), we also detected the other two key genes in ESCs, Sox2 and Oct4, at the same time with Nanog in the ASCs. Whether these two genes are real genes requires further clarification. Thus far, an Sox2 pseudogene has not been reported, but Oct4 pseudogenes have been detected (Suo *et al.*, 2005). Schreiber *et al.* (2013) reported that the existence of multiple pseudogenes can interfere with the evaluation of real genes. Therefore, this matter should be taken into consideration when conclusions are made on the discovery of these key transcriptional factors in certain cell lines, particularly those ESC genes that are found to be expressed in adult stem cells.

Conclusions

We cloned and characterized *Nanog-ps*, a pseudogene of *Nanog*, in sika deer, and we demonstrated that ASCs expressed *Nanog-ps* rather than *Nanog*. *Nanog-ps* retained some characteristics of *Nanog*, such as its nuclear location, and was positively correlated with antler cell stemness; however, no function of Nanog-ps was demonstrated in the present study. Even if it is to be concluded that *Nanog-ps* is functionless in future studies, *Nanog-ps* may still serve as a useful marker for identifying ASCs and evaluating the degree of their stemness.

Acknowledgments

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Chunyi Li, PhD
Chinese Academy of Agricultural Sciences
Institute of Special Wild Economic Animal and Plant Science
No. 4899, Juye Street, Changchun
Jilin 130112
China

E-mail: lichunyi1959@163.com

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