Expression profile of *Nanos2* gene in dairy goat and its inhibitory effect on *Stra8* during meiosis

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Abstract

Objectives: *Nanos2*, an RNA-binding protein, belongs to the *Nanos* gene-coding family and contains two CCHC zinc-finger motifs. In mouse, it plays a pivotal role in male germ cell development, and self-renewal of spermatogonial stem cells. However, little is known of its expression pattern and functions in dairy goat testis.

Materials and methods: Immunohistochemistry and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were used to generate the expression profile of *Nanos2* in dairy goat testis. Furthermore, its overexpression effects on male germline stem cells (mGSCs) were studied using qRT-PCR, immunofluorescence, dual-luciferase reporter assay and western blotting.

Results: *Nanos2* is a conservative gene expressed widely in various tissues, especially in pancreas, and it displays higher expression in adult testes than in other age groups. Overexpression of *Nanos2* significantly downregulated meiosis-related genes, including *Stra8* and *Scp3*, which induced inhibition of meiosis. Results from dual-luciferase reporter assay and western blotting indicated that *Nanos2* directly downregulated *Stra8* in goat GmGSCs.

Conclusions: Taken together, these results suggest that *Nanos2* plays an important role in spermatogonia and that its overexpression restrained meiosis in the dairy goat.

Introduction

During spermatogenesis, spermatogonia undergo the chain of highly organised events of proliferation, self-renewal, differentiation, development, meiosis and morphogenesis, ultimately to form sperm (1). Current research focusses on discovering genes involved in genomics and progress of spermatogenesis, thus exploring interactions of various participatory factors in regulation of spermatogenesis. It is anticipated that such mechanisms will be crucially central issues in genetics and related fields, such as developmental biology and reproductive medicine.

In the mouse testes, there are two types of spermatogonium, undifferentiated and differentiating ones (2). Undifferentiated spermatogonia, also termed spermatogonial stem cells (SSCs), initiate spermatogenesis; they include type Asingle (As, isolated single cell), Apaired (Apr, chains of two cells) and Aaligned (Aal, chains of 4, 8, 16 or occasionally 32 cell) spermatogonia (3). Germ cell-specific protein *Nanos2* is expressed in embryonic germ cells and has an important effect on survival and maintenance of germ cells (4–6). In the post-natal period, spermatogonia recover their proliferative capacity and generate large numbers of differentiating spermatogenic cell populations.

Nanos gene was originally identified in *Drosophila*, where it was found to be required for development of the abdomen. It encodes an RNA-binding protein which has a zinc-finger motif (7). In mouse, *Nanos2* is restricted to germ cells after their localisation in the male gonad. It has a significant impact on sex determination of germ cells by determining male fate while repressing female (8). *Nanos2* has been observed to be expressed in SSCs and is essential for maintenance of the stem cell population during spermatogenesis (9).

Nanos2 expression has been detected in all male gonocytes during embryogenesis, but is limited to spermatogonia after birth (10,11). It is found colonised in

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P-bodies, which are known to act in RNA degradation and storage (12). Interestingly, one further study has reported that *Nanos2* promoted colonisation of CCR4-NOT deadenylation complex to P-bodies, and degraded *Nanos2*-interacting mRNAs *via* a deadenylation-dependent pathway. However, the mechanism of this was not clearly indicated (13).

An optimised method of separation and purification of dairy goat male germline stem cells (GmGSCs) has been established in our laboratory (14). After dissection and chopping the testis, we use a two-step enzyme digestion method. Two kinds of culture protocol, feeder-dependent and feeder-free, have been investigated to grow GmGSCs. Immortalised male goat germline stem cells (GmGSCs-I-SB) were established by transfecting SV40 large T antigen and *Bmi1*, into primary germline stem cells. *In vitro* GmGSCs-I-SB is a proliferative germ cell population, maintained over lengthy periods in feeder-free conditions. They retain their characteristics of germline stem cells by expressing Oct4, GFR α 1, TERT, c-Myc, CyclinD1, Vasa, Dazl and CD90 (15). In this study, we used the feeder-free method to maintain GmGSCs and GmGSCs-I-SB in DM/F12 with 10% FBS.

Nanos2 promotes male germ cell self-renewal by suppression of meiosis (8). Extensive reports have contributed mainly to the understanding of *Nanos2* function in *Mus musculus* (16–18), *Homo sapiens* (19–21), *Danio rerio* (zebrafish) (22) and *Strongylocentrotus purpuratus* (23,24). Up to date, little information on *Nanos2* has been reported in the important field of domestic animals, such as the dairy goat. In this study, we cloned dairy goat *Nanos2* gene and constructed p*Nanos2*-IRES2-AcGFP1 expression vector to monitor expression pattern of *Nanos2* and explore its function during male germ cell meiosis in the dairy goat. This will help further understand molecular mechanisms of spermatogenesis and meiosis in similar mammals.

Materials and methods

Cell and animal collection

The isolation method of primary male goat germline stem cells (GmGSCs) was established in our laboratory, using Guanzhong dairy goat testis (25–27). Immortalised male goat germline stem cells (GmGSCs-I-SB) were established by transfecting SV40 large T antigen and *Bmi1* into primary germline stem cells (15). *In vitro* GmGSCs-I-SB is a proliferative germ cell population, maintained over lengthy periods in feeder-free conditions. Both kinds of cell line were preserved in Shaanxi Centre of Stem Cells Engineering and Technology, Northwest Agriculture and Forestry University.

Guanzhong dairy goat testes, from animals of different (recorded) ages, were collected, specifically from the Yaoan abattoir in Yangling Hi-tech area. All animal procedures were carried out in accordance with provision of the law and approved by the Ethics Committee of Northwest Agriculture and Forestry University on Experimental Animals.

Construction of *Nanos2* eukaryotic expression vector

After *Bos taurus*, *Nanos2* cDNA sequence (XM_002695099.1) in our GenBank, we designed the dairy goat *Nanos2* primers, which were synthesised by Shanghai Sangon Biotech. Upstream primer was 5'-gaAGATCT CAGCTGCTCCTGTCTGCG-3' and downstream primer was 5'-acgcGTCGAC TTGGGAGGGCTG AACCAG-3' (underlined parts are *Bgl*II and *Sal*I restriction sites respectively). *Nanos2* primers are expected to amplify a fragment in the order of 500 bp in length. The fragment was amplified from dairy goat spleen tissue by reverse transcription-polymerase chain reaction (RT-PCR). This procedure was initiated by denaturation at 95 °C for 5 min, then touchdown with 20 cycles of 30 s at 95 °C, 30 s from 65 °C to 55 °C, temperature sequentially decreased by 0.5 °C each cycle, then 40 s at 72 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 58 °C, and 40 s at 72 °C, with final extension for 10 min at 72 °C. Then the RT-PCR product of *Nanos2* was cloned into the pMD18-T vector (TaKaRa, Dalian, China). Subsequently, the *Nanos2* fragment was excised from the vector and inserted into pIRES2-AcGFP1 eukaryotic vector. We named this construct p*Nanos2*-IRES2-AcGFP1. *Bgl*II and *Sal*I restriction sites were multiple cloning sites of pMD18-T and pIRES2-AcGFP1. PCR product was analysed using 2% agarose (Invitrogen, Carlsbad, CA, USA) gel electrophoresis, stained with ethidium bromide (Invitrogen), and visualised under UV illumination (28,29).

Bioinformatic analysis

The dairy goat *Nanos2* gene amplified by RT-PCR, was sequenced by the Shanghai Sangon Biotech Company, and was compared to that of other species, utilising DNAMAN (Lynnon, USA) software; then phylogenetic trees were constructed using MEGA4.1 (Megasoftware, USA) software. Alignment of zinc-finger domains was run in conserved domain database in NCBI.

Cell culture and transfection

Our dairy goat testicular cell lines, GmGSCs and GmGSCs-I-SB, were cultured in DMEM/F₁₂ supplemented with 10% foetal bovine serum (FBS;

HyClone, Logan, UT, USA), 2 mM L-glutamine (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Merk Millipore, Billerica, USA) at 37 °C and 5% CO₂. Culture medium was replaced on alternate days and cells were passaged every 2–3 days, using 0.25% trypsin digestion (Invitrogen).

Cells were transfected with pNanos2-IRES2-AcGFP1 and pIRES2-AcGFP1 in 48-well or 12-well plates using Lipofect-amine™ 2000 (Invitrogen) according to the manufacturer's instructions. After 4–6 h, conditioned medium was changed to DMEM/F₁₂ and cells were further incubated overnight. GFP-positive cells were examined after 24–48 h. Transfected cells were prepared for indirect immunofluorescence (IF), RNA and protein isolation respectively. All experiments were repeated three times.

RT-PCR and qRT-PCR analysis

Total RNA, from dairy goat testes of the different age animals, was extracted using Trizol reagent (TaKaRa). Adult somatic tissues (heart, liver, spleen, lung, kidney, testis and pancreas) and transfected cells were also sampled. Single-strand cDNA was synthesised based on 1 µg RNA, using a reverse transcription kit (Fermentas). qRT-PCR protocol was based on a commercially available kit (Bioer, Hangzhou, China). Reaction conditions were: 94 °C for 5 min, followed by 40 cycles of 20 s at 94 °C, 30 s at 58 °C and 10 s at 72 °C. β-actin was used as internal control to normalise relative expression level of each well. Double ΔCt method was used to measure expression alteration (30). Fluorescence signal was detected every 0.5 °C for 10 s. All gene expressions were analysed using SYBR green and managed with CFX96™ Real-Time System (C1000™; Thermal Cycler) (26,31). PCR primers and length of amplified products are shown in Table 1.

Immunocytochemistry and Immunofluorescent staining

Testicular tissues were derived from goats 2 days post-natal (dpp), 3 M (month), 6 M and 12 M). They were

fixed in 4% paraformaldehyde for 24 h, then embedded in paraffin wax and sectioned at 5 µm. Sections were deparaffinised, rehydrated in a series of ethanols and subjected to epitope retrieval using standard methods. Specimens were then incubated in 3% H₂O₂ for 10 min to inhibit endogenous peroxidase activity. Sections were blocked for non-specific binding, with 4% goat normal serum and incubated in primary antibody (anti-Nanos2, 1:200; Abcam, Cambridge, United Kingdom) overnight at 4 °C. Slides were washed in PBST, incubated in appropriate HRP-conjugated secondary antibody and developed using DAB detection reagents (Beijing Zhongshan Golden Bridge Biochemical Factory, Beijing, China). Concurrently, negative controls were incubated in HRP-conjugated secondary antibodies alone (27).

After 48 h transfection, heterogeneous cell populations (dairy goat mGSCs and mGSCs-I-SB) in 48 wells were fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature, then permeabilised with 0.1% Triton X-100 for 10 min, followed by washing three times in PBS. After blocking in 1% BSA (Sigma-Aldrich, St. Louis, USA) at room temperature for 30 min, cells were incubated in primary antibodies overnight at 4 °C. The following antibodies were used: anti-Nanos2 (1:300; Abcam), anti-Stra8 (1:500; Abcam), and anti-Scp3 (1:200; Santa Cruz, Dallas, USA). After three washes in PBS, cells were incubated in goat anti-rabbit IgG conjugated to AlexaFluor 594 (Invitrogen) for 1 h in the dark at room temperature, followed by three washes in PBS, then incubated in DAPI (4',6-diamidino-2-phenylindole; Sigma) at room temperature for 3 min. Images were captured and analysed using a Leica fluorescence microscope (32).

Western blotting

Total protein of mGSCs and mGSCs-I-SB transfected with or without Nanos2 were extracted in 1× SDS-PAGE sample loading buffer. Proteins were loaded into wells of SDS-PAGE gel, then run for 1–2 h at 100 V. After transferring to PVDF membranes, samples were incubated in primary antibodies, including anti-β-actin (1:1000;

Table 1. Primer sequences and PCR reaction conditions

Gene	Primer	T _m (°C)	Size (bp)
β-actin	Forward: GCGGCATCCACGAACTAC	58	104
	Reverse: TGATCTCCTTCTGCATCCTGTC		
Nanos2	Forward: GGAAGGACTACTTCAACCTGAGCC	58	120
	Reverse: ATAATGCCGAGAATGGGACAC		
Stra8	Forward: AAGGACAGCGGGTTGAC	58	170
	Reverse: TCGGGTTTTTTTGAGTTGC		
Scp3	Forward: GTATGGAGGACTTGGAGA	58	138
	Reverse: GAGACTTTCGGACACTTGC		

Beyotime, Haimen, Jiangsu, China) and anti-Stra8 (1:1000; Abcam). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as secondary antibody (1:1000; Beyotime). Results were detected using Thermo Scientific Pierce ECL western blotting substrate (Thermo Scientific, Pittsburgh, USA) and analysed using Tanon-410 automatic gel imaging system (Shanghai Tianneng Corporation, China) (26,28).

Dual-luciferase reporter assay

Our group has constructed the psiCHECK-2-Stra8 recombinant plasmid, which has been inserted into Stra8 mRNA 3'-UTR (Jiang Wu, unpublished data). Hela cell line, GmGSCs and GmGSCs-I-SB were transfected with 250 ng psiCHECK-2-Stra8 and 250 ng pNanos2-IRES2-AcGFP1 or pIRES2-AcGFP1 with Lipofectamin 2000 reagent (Invitrogen) according to the manufacturer's instruction. Twenty four hours later, cells were lysed in passive lysis buffer for dual-LUC assays (vigorous). Ratio of Renilla luciferase RLU to Firefly luciferase RLU represented degree of Stra8 3'-UTR activation. Each experiment was repeated at least three times.

Statistical analysis

Data are presented as mean $\pm$ SD and standard deviation (SD) in this study was calculated in three replicates.

Differences in expression of specific markers was evaluated using one-way analysis of variance (one-way ANOVA) using Graph pad Prism (GraphPad Software, San Diego, USA) software; $P < 0.05$ was considered as statistically significant.

Results

Expression profile of *Nanos2* in dairy goat

Nanos2 gene was detected in the tissues of dairy goat by qRT-PCR. We found that it was not only expressed in mature dairy goat testis but also in adult somatic tissues, heart, liver, spleen, lung, kidney and pancreas (Fig. 1a); it was highly expressed in pancreas and spleen, but relatively lower in liver. These results also indicate that *Nanos2* was not testis-specific here, but is ubiquitous as far as we investigated; it thus may play an important role in these tissues in dairy goat. Moreover, *Nanos2* mRNA level was high in adult testis compared to other age testis, and intersex testis (Fig. 1b). As shown in Fig. 1c, NANOS2 had higher protein level expression in adult testis than the other age groups (Fig. 1c). This result was consistent with *Nanos2* expression tendency at mRNA level in different age testes. Not surprisingly, we could see NANOS2 located in cytoplasm of spermatogonia near basement membranes of seminiferous tubules (Fig. 1c black arrows); this coincides with that of mouse.

Figure 1. Expression profile of *Nanos2* gene in the dairy goat. (a) *Nanos2* expression in various tissues of dairy goat detected by qRT-PCR. (b) qRT-PCR analysis of *Nanos2* expression in dairy goat testis at different ages and male intersex goat. (c) Expression of *Nanos2* in dairy goat testes, at different ages, by immunohistochemistry. Error bars indicate the SD values of technical duplicates of qRT-PCR results. P values for qRT-PCR were obtained using one-way analysis of ANOVA. * $P < 0.05$ (scale bar = 50 μ m).

Identification and bioinformatic analysis of Guanzhong dairy goat *Nanos2*

Nanos2 CDS was amplified from dairy goat spleen tissue by RT-PCR, and primers were designed based on predicted bovine *Nanos2*. We obtained a fragment of 558 bp as expected from dairy goat spleen tissue by RT-PCR (Fig. 2a). After cloning into pMD18-T vector for sequencing, the correct clone was excised and inserted into pIRES2-AcGFP1 eukaryotic vector. We utilised double digestion of *Sal*I and *Bgl*II to identify accuracy of recombinant vector and named the plasmid identified correctly as p*Nanos2*-IRES2-AcGFP1 (Fig. 2b). The cloned goat *Nanos2* gene was analysed by sequencing (Fig. 2c). We found it to have high sequence similarity to corresponding homologous regions of *Bos taurus*, *Callithrix jacchus*, *Mus musculus*, *Canis familiaris* and *Pan troglodytes* respectively. Then, we constructed the phylogenetic tree using MEGA4.1 software (Fig. 2d). Meanwhile, we also found the conservative zinc-finger domain located at amino acid 63–117 of dairy goat *NANOS2* by amino acid sequence alignment, in the conserved domain database of NCBI (Fig. 2e). We concluded that dairy goat *NANOS2* had the prerequisite to bind target mRNA through its CDS and protein alignments among species. Dairy goat *Nanos2* was uploaded to NCBI and obtained the accession no. KC155624.1.

Overexpression of *Nanos2* in GmGSCs and GmGSCs-I-SB

Recombinant plasmid p*Nanos2*-IRES2-AcGFP1 and control vector pIRES2-AcGFP1 were transfected into dairy goat mGSCs and mGSCs-I-SB respectively. GFP-positive cells were observed in both groups by 24 h

after transfection, indicating that vectors were successfully transfected (Fig. 3a,c). Transfection efficiency of GmGSCs was higher than that of GmGSCs-I-SB, in the order of 30% and 20% respectively (Fig. S1). Here, although transfection efficiency was not the same (which may lead to different changed degrees), variation tendency was eventually consistent. Forty eight hours after transfection, total RNA and protein were extracted from the heterogeneous cell populations. As shown in Fig. 3b and 3d, *Nanos2* expression level was significantly higher in p*Nanos2*-IRES2-AcGFP1-transfected groups than pIRES2-AcGFP1 groups, as shown by qRT-PCR analysis (Fig. 3b,d). Furthermore, expression of *Stra8* and *Scp3* (markers of meiosis), were significantly downregulated in the *Nanos2* overexpression group compared to controls (Fig. 3b,d). Similarly, expression of *Stra8* and *Scp3* were reduced at protein levels (Figs 4,S2 purple dashed boxes), while *NANOS2* expression was upregulated after transfection with p*Nanos2*-IRES2-AcGFP1 (Figs 4,S2 white dashed boxes).

Nanos2 inhibited *Stra8* expression by directly binding to *Stra8* mRNA 3'-UTR

Stra8 was significantly downregulated at the protein level by 48 h after *Nanos2* transfection, compared to controls, analysed by western blotting. In GmGSCs, protein expression of *Stra8* was attenuated by 30% (Fig. 5a,b), meanwhile being reduced by 50% in GmGSCs-I-SB (Fig. 5c,d). This indicated that *Nanos2* seemed to block the process of meiosis by suppression of expression of *STRA8*. To explore the relationship between *Nanos2* and *Stra8*, we carried out the dual-luciferase

Figure 2. Identification and bioinformatic analysis of Guanzhong dairy goat *Nanos2* gene. (a) Electrophoretogram of PCR product of *Nanos2* CDS (558 bp). (b) Restriction enzyme digestion analysis of p*Nanos2*-IRES2-AcGFP1 and pMD18-T-*Nanos2*. Lane M1: DNA ladder of 2000 bp. Lane M2: DNA ladder of 10 000 bp. (c) Sequence of Guanzhong dairy goat *Nanos2*. (d) Phylogenetic trees of *Nanos2* sequence in various species. (e) Sequence alignment of *Nanos* protein from various species to highlight the conserved zinc-finger domain.

Figure 3. Overexpression of *Nanos2* in GmGSCs and GmGSC-I-SBT. (A, C) GFP-positive GmGSCs and GmGSCs-I-SBT were observed by fluorescence microscopy. (a' and b'): Cells transfected with pIRES2-AcGFP1 (a', Phase contrast; b', fluorescence microscopy); (a and b): Cells transfected with pNanos2-IRES2-AcGFP1 (a, Phase contrast; b, fluorescence microscopy). (B, D) mRNA expression level of *Nanos2*, *Stra8* and *Scp3* in pNanos2-IRES2-AcGFP1 transfection group in GmGSCs and GmGSCs-I-SBT were analysed by qRT-PCR. Error bars indicate SD values of technical duplicates of qRT-PCR results. *P* values for qRT-PCR were obtained using one-way analysis of ANOVA. **P* < 0.05 (scale bars = 200 μ m).

experiment. From these results we found that *Nanos2* bound directly to *Stra8* 3'-UTR in GmGSCs (Fig. 5f), but not in HeLa cells and GmGSCs-I-SBT (Fig. 5e,g). Although NANOS2 bound the 3'-UTR of *Stra8* directly in GmGSCs, there was no similar phenomenon in HeLa cells or GmGSCs-I-SBT. This difference might be caused by cell specificity and complexity of *Nanos2* regulation function on *Stra8*.

Discussion

It has been previously documented that Nanos is essential for functional germ cell formation in *Drosophila* (33). *Nanos2*, a member of the Nanos family, plays a pivotal role in sexual differentiation of mouse germ cells during embryogenesis and maintenance of spermatogonial stem cell populations after birth (13). However, up to now, the exact function of *Nanos2* in germ cells and SSCs in the dairy goat had remained unclear. In this study, we cloned dairy goat *Nanos2* CDS and constructed pNanos2-IRES2-AcGFP1 eukaryotic expression

vector. Meanwhile, we explored regulatory effects of *Nanos2* on *Stra8*.

We found that the dairy goat *Nanos2* sequence had high similarity to that of *Bos taurus*, *Callithrix jacchus*, *Mus musculus*, *Canis familiaris*, and *Pan troglodytes*, which further suggests that it is highly conservative across species. *Nanos2*, an RNA-binding protein, contains a zinc-finger domain conserved in the Nanos family. It is widely expressed in many species from *Drosophila* to humans (13). In this study, we found conserved zinc-finger domains in dairy goat *Nanos2* protein by means of amino acid sequence alignment with that of other species. Although the function of Nanos family members was conservative, homology outside the zinc-finger domain was relatively low, which might lead to different functions of *Nanos2* in specifying and maintaining germ cell development across species (34,35).

In mouse testes, *Nanos2* expression is limited to a subset of undifferentiated spermatogonia, particularly As and Apr types, after birth (6), and expression of *Nanos2* cannot be detected in E12.5 mouse somatic cells (5).

Figure 4. Immunofluorescence staining of GmGSCs after overexpression of *Nanos2*. GFP', *Nanos2*' (red), *Stra8*' (red), *Scp3*' (red), DAPI' and Merge': Control transfected with pIRES2-AcGFP1. GFP (green), *Nanos2*, *Stra8* and *Scp3* (red), DAPI (nuclei, blue) and Merge: Cells transfected with p*Nanos2*-IRES2-AcGFP1. White dashed boxes indicate the expression of *Nanos2* was upregulated. Purple dashed boxes mean the *Stra8* and *Scp3* were depressed in the *Nanos2*-upregulated cells. Blue dashed boxes show no changes in non-GFP cells (scale bars = 200 μ m).

Figure 5. *Nanos2* suppressed *Stra8* expression by binding to the 3'-UTR of *Stra8* mRNA in GsGSCs and GsGSCs-I-SB. (a–d) Detection of *Stra8* expression in GmGSCs (a and b) and GmGSCs-I-SB (c and d) after *Nanos2* overexpression, by western blotting. (b, d) Image quantitation of (a) and (c) respectively. (e–g) Dual-luciferase activity analysis after transfection overexpression of *Nanos2* and *Stra8* 3'UTR into HeLa cell line, GsGSCs, GsGSCs-I-SB. Error bars indicate the SD values of technical duplicates of Gray value and RLU results. *P* values for western blotting and dual-luciferase experiments were obtained using one-way analysis of ANOVA (* $P < 0.05$).

However here, we observed that *Nanos2* was also expressed in various goat somatic tissues, besides testis, furthermore relatively highly in spleen and pancreas. This suggests that *Nanos2* might have an influence on multiple tissues of dairy goats, especially in spleen and pancreas. We cannot ignore high expression level of *Nanos2* in spleen, particularly since we easily cloned *Nanos2* CDS from cDNA of spleen, but not testes. This may relate to differences between species – mouse belonging to the rodentia, while dairy goats are artiodactyla; some functions of some genes may have been changed during the process of evolution. In addition, in dairy goat testis, we observed that *Nanos2* expression was higher in adults than other ages. Thus, we deduced that it might still exert an important effect on spermatogenesis in this species.

Nanos2 is required absolutely for spermatocyte meiosis in mouse (36). Loss-of-function of *Nanos2* has resulted in deficiency of spermatogonial cell population and subsequently causes germ cell depletion, indicating *Nanos2* to be required for spermatogonial stem cell maintenance (4). In this study, summarising the results of overexpression in GmGSCs and GmGSCs-I-SB, we first showed that *Nanos2* downregulated expression of *Stra8* and *Scp3*, markers of meiosis in dairy goat male germ cells (37,38). *Stra8*- and *Scp3*-positive meiotic cells were reduced in number in *Nanos2*-overexpression cells, indicating that it prevented entry of spermatogonia into meiosis, which is consistent with results in mouse (13). Overexpression of *Nanos2* significantly reduced meiosis-related gene expression suggesting that it played

a significant role in meiosis process of male germ cells. Concerning Fig. 3b, expression of *Stra8* mRNA has no significant change in GmGSCs, but protein level was reduced significantly. It could be that overexpression *NANOS2* bound to *Stra8* mRNA and inhibited its translation, but does not introduce degradation of mRNA. High-passaged GmGSCs may be the critical point because the primary GmGSCs can only be passaged in vitro for six to seven generations. Physiological condition of GmGSCs changes along with passage.

In mouse, *NANOS2* must interact with pumilio 2 (*PUM2*) to bind target mRNA, then transfer to P-bodies; the mRNA is subsequently degraded by CCR4/NOT1 deadenylase complex (39–41). This means that some important cofactor is essential during binding of *NANOS2* to target mRNA. This might explain why *Nanos2* overexpression in HeLa cells did not lead to reduction in relative Firefly/Ranilla luciferase RLU. Concerning GmGSCs-I-SB, its physiological characteristics could have been changed to be short of the cofactor interacting with *Nanos2* during the process of immortalisation, so that exogenous *Stra8* could not be suppressed. To explore which components are needed during suppressing *Stra8* expression by *Nanos2* in germ cells requires more study.

In conclusion, we analysed expression pattern of *Nanos2* in dairy goat testis and found it to be a conserved gene. Its overexpression in GmGSCs and GmGSCs-I-SB resulted in reduction in expression of meiosis-related genes. *Nanos2* exerted a significantly inhibitory effect on *Stra8* expression by binding its 3'-

UTR in germ cells. Therefore, we assume that *Nanos2* is required during spermatogenesis and meiosis in dairy goat testis. This study established a theoretical foundation for further investigating the mechanism of *Nanos2* in spermatogenesis in domestic animals and might provide a potential target gene for treating male infertility.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Transfection efficiency of GmGSCs and GmGSCs-I-SB.

Fig. S2. Immunofluorescence staining of GmGSCs-I-SB after overexpression of *Nanos2*.