Expression pattern of Ngn3 in dairy goat testis and its function in promoting meiosis by upregulating Stra8

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Received 27 July 2013; revision accepted 19 September 2013

Abstract

Objectives: Ngn3 is a typical transcription factor and marker of differentiating spermatogonial stem cells (SSCs) in mouse, belonging to the basic helix-loop-helix (bHLH) family. Its gene is specifically expressed in A type spermatogonia in mouse testis, thus plays a critical role in controlling differentiation of SSCs. However, roles of *Ngn3* and its protein in dairy goat testis remain unknown.

Materials and methods: Testis development and expression patterns of Ngn3 were analysed by immunofluorescence and quantitative reverse transcription-polymerase chain reaction (QRT-PCR) in the dairy goat. Furthermore, effects of its overexpression on male germline stem cells (mGSCs) were evaluated by QRT-PCR, immunofluorescence, luciferase reporter assay and western blotting.

Results: Revealed that Ngn3 was expressed more highly during puberty and in the adult than in testis of other ages. Overexpression of Ngn3 promoted expression of meiosis-related gene *Stra8* and stem-cell differentiation marker CD117, but suppressed expression of *Plzf*, a classical marker of SSCs. Furthermore, Ngn3 did not promote expression of *Stra8* directly as shown in transcription and translation levels detected by luciferase reporter assay and western blotting.

Conclusions: Taken together, these results suggest that Ngn3 plays an important role in spermatogenesis and that overexpression of Ngn3 can promote meiosis in testis of the dairy goat.

Introduction

Spermatogenesis is responsible for maintaining male fertility from puberty to maturity, involving complex self-renewal and differentiation of SSCs (1). In mammalian testis, there are two kinds of spermatogonium, undifferentiated spermatogonia and differentiating spermatogonia (2). Undifferentiated spermatogonia – spermatogonial stem cells (SSCs), consist of A_{single} (A_s), A_{paired} (A_{pr}) and A_{aligned} (A_{al}) spermatogonia. A_{pr} and A_{al} differentiate into B type spermatogonia, then form mature sperm after meiosis (3,4).

Ngn3 is a typical transcription factor and marker of differentiating SSCs (5,6), belonging to the class B basic helix-loop-helix (bHLH) transcription factor family (7,8). It has been reported that expression of Ngn3 increases gradually in mouse mature testis (8), as differentiation of A_s and Ngn3-positive germ cells gives rise to spermatogenesis. Recent studies have shown that STAT3 directly regulates transcription of *Ngn3* gene (9), and GDNF (glial cell line-derived neurotrophic factor) restrains expression level of Ngn3 by suppressing phosphorylation of STAT3 (10,11). GDNF is a requisite factor in maintaining the undifferentiated state of SSCs. The basic helix-loop-helix (bHLH) transcription factor family plays critical roles in regulating differentiation processes in many tissues, such as skeletal muscle, nerve and pancreas (7,12–17). Usually, these transcription factors form homodimer or heterodimer complexes with a further member of bHLH transcription factor family to regulate transcription of target genes (18,19).

In mammals, the process of spermatogenesis requires retinoic acid (RA) and its target gene *Stra8* (stimulated

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by retinoic acid 8) (20,21). *Stra8* was first described as an RA-responsive gene in 1996 (22). In both ovary and testis, expression of *Stra8* is controlled by presence of RA and its concentration, so that meiosis becomes initiated at different time points (23–25). CD117 is a differentiation marker of spermatogonia; CD117-negative spermatogonia have high spermatogenic colony-forming activity after being transplanted into germ cell-deficient testes (26,27). Plzf, a transcription repressor expressed in spermatogonia, maintains self-renewal of SSCs (28) and has commonly been used as a distinctive marker of undifferentiated spermatogonia.

Until recently, most studies related to Ngn3 have been carried out on *Mus musculus* (13,29,30), *Homo sapiens* (31,32) and *Danio rerio* (33,34) with only few reports related to livestock. The dairy goat can be considered to be a kind of large-scale experimental animal, not only does it produce nourishing milk and wonderful meat but since it has a relatively short length of gestation, early sexual maturation and due to its smaller size than cattle, it can be used for studying lactation, ruminant nutrition and foetal development (30,35).

Ngn3-positive germ cells give rise to spermatogenesis, having potential roles in regulating dairy goat SSCs differentiation, thus it is necessary to expound what roles Ngn3 plays in dairy goat testis. In this study, we first explored expression pattern of Ngn3 in the dairy goat and cloned the coding sequence of *Ngn3* by PCR; then we constructed the Ngn3 recombinational eukaryotic plasmid to study its effects on meiosis in dairy goat male germline stem cells (mGSCs).

Materials and methods

Animals and cells

Primary male goat germline stem cells (GmGSCs) were established from Guanzhong dairy goat testis (35–37). Immortalized male goat germline stem cells (GmGSCs-I-SB) were established by transfecting SV40 large T antigen, Bmi1 into primary germline stem cells, which can be maintained *in vitro* for long periods in appropriate culture conditions, as a proliferative germ cell population (36). Both types of cell line were stored in Shaanxi Centre of Stem Cells Engineering and Technology, Northwest A&F University. Guanzhong dairy goat testes from different age animals were supplied by Yaoan slaughterhouse in the Yangling Agriculture and Hi-tech Area. All animals were maintained, operated on, killed and dissected according to the guidelines of Northwest A&F University. Thirty days postnatal (dpp) to 6-month-old goats were defined as being pre-pubertal (37).

Immunofluorescence staining

Testicular tissues derived from 2 days postnatal (dpp), 90 dpp, and adult dairy goats were fixed in 4% paraformaldehyde and embedded in paraffin wax, respectively. Embedded blocks were sectioned at 5 µm and samples were immersed in xylene twice, (8 min); 100%, 95%, 75% alcohols respectively (5 min) and thereafter rinsed in deionized water twice for 5 min. Slides were then soaked in boiling citrate buffer for 15–25 min, and when cooled to room temperature, were washed three times in PBS (5 min). Then samples were blocked in blocking solution (PBS+1%BSA) for 30 min and incubated with primary antibody against Ngn3 (1:100; Abcam, Cambridge, UK) overnight at 4 °C. Sections were then washed in PBS three times, and incubated in goat anti-rabbit IgG antibody conjugated to AlexaFluor 488 (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature, followed by mounting with coverslips on to glass slides with Vectashield mounting medium containing DAPI (Vector Laboratories, Servion, Switzerland). Concurrently, negative controls were stained with conjugated secondary antibodies alone. Images were captured using a Leica fluorescence microscope.

Construction of recombination plasmid

Dairy goat Ngn3 primers were designed according to published *Bos taurus* Ngn3 mRNA sequence (XM_591702.3). Ngn3 is 867 base pair (bp) and primers were synthesized (SanBoYuanZhi Company, Peking, China):

Ngn3: Forward: 5'-CGGAATTCCCGACCCACTCTTACCCTTACC-3';

Reverse: 5'-GCGTCGACCAGGAGGAACAACACTAGGTTTGAGAG-3'.

Genes were amplified from the dairy goat genome by PCR, the genome having been obtained from GmGSCs-I-SB using TIANamp Genomic DNA Kit (Tiangen, Peking, China). PCR steps included denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, and final extension at 72 °C for an additional 10 min. Specific fragments were then cloned into pMD18-T cloning vector (TaKaRa, Dalian, China). After white-blue screening, positive clones were identified by PCR, restriction enzyme digestion and sequencing. Fragments were then cloned into pIRES2-AcGFP1 to obtain pNgn3-IRES2-AcGFP1 recombination plasmid. The plasmid was verified by PCR and restriction enzyme digestion. PCR products were analysed in 1% agarose (Invitrogen) gel electrophoresis, stained with ethidium bromide (Invitrogen) and visualized under UV illumination.

Bioinformatics analysis

The Guanzhong dairy goat Ngn3 CDS was obtained and sequenced by SanBoYuanZhi Company. The sequence was then aligned with Ngn3 sequences of further species in Genbank using DNAMAN software, and the phylogenetic tree was depicted with MEGA4.1. The protein domain of NGN3 was predicted by SWISS-MODEL Workspace website and RasMol software. Alignment of HLH protein was run in conserved domain database in NCBI.

Cell transfection

The pNgn3-IRES2-AcGFP1 recombination plasmid and pIRES2-AcGFP1 were transfected into GmGSCs and GmGSCs-I-SB in 48-well or 12-well plates using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's instructions. GFP-positive cells were examined after 24 h, and total RNAs from the two groups were extracted respectively, after plasmids were transfected for 48 h, to test levels of mRNA of Stra8, Plzf and CD117, by RT-PCR and QRT-PCR (38).

Fluorescence immunocytochemistry

Transfected GmGSCs and GmGSCs-I-SB at 48 h were fixed in 4% paraformaldehyde for 15 min and treated with 0.2% Triton X-100 for 10 min at room temperature. Non-specific antibody binding was blocked by incubating them in 1% BSA in PBS for 30 min at room temperature. Ngn3 (1:100; Abcam), Stra8 (1:300; Abcam), CD117 (1:200; Biolegend, San Diego, CA, USA), Plzf (1:200; Santa Cruz, Dallas, TX, USA) were added to the cells and incubated overnight at 4 °C. After being washed three times in PBS, specimens were incubated in goat anti-rabbit IgG antibody conjugated to AlexaFluor 594 (Invitrogen) for 1 h at room temperature in the dark. Images were captured by a Leica fluorescence microscope.

RT-PCR and quantitative RT-PCR analysis

Total RNA was extracted from different tissues of the dairy goats, including testis, at 2 dpp, 120 dpp, 150 dpp, 180 dpp, 210 dpp, and adult, using TRIzol reagent (Tiangen) according to the manufacturer's instructions, and RNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA). cDNA was synthesized based on 1 µg RNA, using a commercially available kit (TaKaRa). RT-PCR primers used are listed in Table 1, all markers of germ cells or mGSCs.

Relative *Ngn3*, *Stra8*, *CD117* and *Plzf* gene expression levels were determined using a CFX96™ Real-Time

Table 1. Primer sequences

Name	Sense primer	Antisense primer
β -actin	5'-acggcatcgtcaccaact-3'	5'-aggaaggaaggctggaagag-3'
Ngn3	5'-gcgagttggcactgagca-3'	5'-aagctgtgtccgctatgc-3'
Stra8	5'-cgccatctcccatctgtg-3'	5'-tgtcttcacgtgccttc-3'
CD117	5'-tcccaaacctcaacaccgacag-3'	5'-gtgtaagtgcctcctcagtc-3'
Plzf	5'-caccgaacagccagcactat-3'	5'-cagcgtacagcaggtcatccag-3'

System (C1000™; Bio Rad Thermal Cycler, Foster City, CA, USA). Quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) was set up in 15 µl reaction mixtures containing ddH₂O 6.3 µl, 2× SYBR (Bioer Co. Ltd., Hangzhou, China) 7.5 µl, cDNA 0.5 µl, sense primer 0.3 µl, antisense primer 0.3 µl, TaqDNA polymerase 0.1 µl. Reaction conditions were as follows: 95 °C for 5 min, and then 40 cycles 95 °C for 20 s, 58 °C for 30 s and 70 °C for 10 s. To make quantitative comparisons between samples, we normalized *Ngn3*, *Stra8*, *Plzf* and *CD117* gene expressions to expression of β -actin in each well. Double Δ Ct method was used to measure expression alteration (38); fluorescence signal was collected every 0.5 °C for 10 s.

Luciferase reporter assay

Here, we used pStra8-LUC reporter vector. This had been recombined in our centre, in which the 1.4 kb fragment of Stra8 promoter was inserted into SacI/HindIII sites of pGL3-basic vector (Clontech Laboratories, Inc., Mountain View, CA, USA) (36). 250 ng pStra8-LUC, pNgn3-IRES2-AcGFP1 or pIRES2-AcGFP1 (negative control) was co-transfected into HeLa cells, GmGSCs and GmGSCs-I-SB, in 48-well plates using Lipofectamine2000 (Invitrogen). After 48 h, target validation assays were performed using the luciferase reporter system (Galen Biopharm International Co., Ltd, Souderton, PA, USA) according to the manufacturer's instructions.

Western blotting

Total cell extracts were prepared from GmGSCs and GmGSCs-I-SB transfected Ngn3 or non-transfected, and proteins were extracted in 1× SDS-PAGE sample loading buffer. Total proteins were resolved by SDS-PAGE, transferred to PVDF membrane, and probed with β -actin (1:1000; Beyotime, Haimen, Jiangsu, China), and Stra8 (1:1,000; Abcam); horse-radish peroxidase-conjugated anti-mouse and anti-rabbit was used as secondary antibody (1:1,000; Beyotime), respectively. Detection was performed using Thermo Scientific Pierce ECL western blotting substrate (Thermo Scientific) (39). Results were

analysed using Tanon-410 automatic gel imaging system (Shanghai Tianneng Corporation, Shanghai, China).

Statistical analysis

Data are presented as mean $\pm$ SEM. Differences in expression of specific markers were evaluated using Student's *t*-test (Excel, Microsoft Corporation, Redmond, WA, USA). Results of different treatments were considered significantly different when *P* values <0.05 were obtained.

Results

Expression pattern of Ngn3 in the dairy goat

QRT-PCR analysis showed that Ngn3 was widely expressed over the range of adult dairy goat organs sampled, including heart, liver, spleen, lung, kidney, pancreas and testis. Among them all, Ngn3 expression level in heart and lung were significantly higher than in the others, while testes had the lowest (Fig. 1a). Ngn3 mRNA levels in adult testis were higher than those of neonatal and pubertal testes (Fig. 1b). Concerning Ngn3 protein expression level, results of immunofluorescence had a similar pattern to translation and transcription levels of Ngn3; its translational abundance in pubertal and adult testes was significantly higher than in foetal testis

(Fig. 1c, Fig. S1). In addition, Ngn3 protein was located in cytoplasm of spermatogonia and percentage of Ngn3-positive spermatogonia in puberty and adult testes was higher than in foetal testis.

Cloning and bioinformatics analysis of dairy goat Ngn3

Guanzhong dairy goat Ngn3 coding sequence contained one exon only, thus it was cloned by PCR from the adult goat genome. The PCR product 867 bp (Fig. 2a), was identified by *EcoRI*, *SalI* restriction enzyme digestion (Fig. 2b) and sequencing (Fig. 2c); this sequence was submitted to NCBI, and obtained a formal sequence number, KC154005.1. We then aligned the CDS of goat Ngn3 with the homologous sequences of *Bos taurus*, *Homo sapiens* and other species by using DNAMAN software and results showed that there was a high degree of sequence similarity in Ngn3 CDSs among the different species, CDS of dairy goat Ngn3 having 100% similarity to that of *Bos taurus* (Fig. 3a). This indicates that Ngn3 has been a conserved gene over evolution. The phylogenetic tree was constructed by making a homology comparison of Ngn3 CDSs between various species using MEGA4 (Fig. 3b). We then put Ngn3 amino acid sequence into SWISS-MODEL Workspace website to seek a typical domain; finally, we found a typical HLH model, 2ql2B (Fig. 3c(a)), and predicted the HLH domain of Ngn3 (Fig. 3c(b)). To ensure reliability of the

Figure 1. Expression pattern of Ngn3 in Guanzhong dairy goat testis. (a) QRT-PCR analysis has shown that Ngn3 was highly expressed in the heart, lung and pancreas. (b) At different ages of animal, level of Ngn3 of normal adult was significantly higher than of younger and male intersex testes, analysed by QRT-PCR. (c) Ngn3 was significantly higher in puberty and adult testes analysed by immunofluorescence. Gray arrows indicate false-positive Leydig cells, and yellow arrows represent positive spermatogonia. Dotted line indicate seminiferous tubules. Scale bar = 100 μ m.

Figure 4. Overexpression of Ngn3 in GmGSCs and GmGSCs-I-SB. (a, b): GmGSCs were transfected with pIRES2-AcGFP1 (a, phase contrast; b, fluorescence microscope); (c, d): GmGSCs were transfected with pNgn3-IRES2-AcGFP1 (c, phase contrast; d, fluorescence microscope). Scale bar = 200 μ m. (a, b): GmGSCs-I-SB were transfected with pIRES2-AcGFP1 (a, phase contrast; b, fluorescence microscope); (c, d): GmGSCs-I-SB were transfected with pNgn3-IRES2-AcGFP1 (c, phase contrast; d, fluorescence microscope). Scale bar = 200 μ m. (c) Expression level of *Ngn3* in pNgn3-IRES2-AcGFP1 transfected group in GmGSCs was significantly higher compared to pIRES2-AcGFP1 group analysed by QRT-PCR. Additionally, expression of meiosis gene *Stra8* and germ cell differentiation marker CD117 were remarkably upregulated in pNgn3-IRES2-AcGFP1 transfected group. In contrast, expression of *Plzf*, which maintains self-renewal of germ cells, was suppressed. (d) By QRT-PCR analysis, there was the same trend relating to *Stra8*, CD117 and *Plzf* in transfected pNgn3-IRES2-AcGFP1 GmGSC-I-SB. * $P < 0.05$

groups. Meanwhile, expressions of differentiation markers *Stra8* and CD117 were higher; however, self-renewal marker *Plzf* was lower (Fig. 4c,d). *Ngn3* and *Stra8* proteins were found to be located in the cytoplasm of GmGSCs and GmGSC-I-SB (Fig. S2), while in overexpressed *Ngn3* GmGSCs and GmGSCs-I-SB, *Ngn3* was found to have translocated into the nucleus only in a minority of cells with high intensity GFP. Simultaneously, translocation of *Stra8* from cytoplasm to nucleus was only observed in a few GmGSCs-I-SB with highly overexpressed *Ngn3* gene. These results demonstrated that *Ngn3* was significantly upregulated in the two cell lines, and *Ngn3* protein could promote *Stra8* protein to translocate to the nucleus. Expression of both *Stra8* and CD117 was stimulated (Figs 5,6).

Ngn3 promoted Stra8 indirectly

To explore the relationship between *Ngn3* and *Stra8*, we proceeded with luciferase reporter assay and wes-

tern blotting. First, we tested the effects of *Ngn3* on the promoter of *Stra8* in HeLa cells, GmGSCs and GmGSCs-I-SB in turn (Fig. S3). In HeLa cells, no significant change detected, but in GmGSCs and GmGSCs-I-SB, abundance of luciferase with *Stra8* promoter was significantly higher (Fig. 7a–c). HeLa cells, an immortal cell line, was derived from cervical cancer cells taken on February 8, 1951 http://en.wikipedia.org/wiki/HeLa-cite_note-pmid13052828-2#cite_note-pmid13052828-2 (40). They do not contain the set of elements required to promote *Stra8*; however, as male germline stem cells, GmGSCs and GmGSCs-I-SB provided an environment in which to transmit the signal from overexpression of *Ngn3* to upregulated *Stra8*. All the above demonstrated that *Ngn3* function affected *Stra8* promoter indirectly. Then, quantification of change of *Stra8* protein level showed that it also was significantly increased by overexpression of *Ngn3* – by 36% compared to empty vector group in GmGSCs and 38% in GmGSCs-I-SB (Fig. 7d–f).

Figure 5. Immunofluorescent staining of GmGSCs after overexpression of Ngn3. Only in these cells with high-intensive green fluorescence (purple arrows), was Ngn3 translocated to the nucleus (yellow arrows). Stra8, CD117 and Plzf, demonstrated no clear change. Scale bar = 200 μ m.

Figure 6. Immunofluorescent staining of GmGSCs-I-SB after overexpression of Ngn3. Only in these cells with high-intensive green fluorescence (purple arrows), was Ngn3 translocated to the nucleus (yellow arrows). Concerning Stra8, in cells with high-intensive green fluorescence (left red arrow), Stra8 protein had translocated to the nucleus (left white arrow). Right red and white arrows were controls for cells with weak-intensive green fluorescence; Stra8 protein had not been obviously affected. Scale bar = 200 μ m.

Discussion

This study for the first time obtained the CDS region and expression pattern of Ngn3 in the dairy goat, and clarified the role of Ngn3 in the process of spermatogen-

esis by overexpression of Ngn3. Ngn3, also known as NEUROG3, is the third member of the neurogenin family (13–15), and as a heterodimer transcriptional regulator, it initiates transcriptional activation of NEUROD1,

Figure 7. Ngn3 had an indirect effect on Stra8. (a) level of Stra8 did not change significantly in the pNgn3-IRES2-AcGFP1 group compared to controls, analysed by pStra8 promoter-LUC assay in HeLa cells. (b) In GmGSCs, level of luciferase was 70% higher in the pNgn3-IRES2-AcGFP1 group compared to the control group. (c) In GmGSCs-I-SB, level of luciferase was 120% higher. (d) Protein level of Stra8 was also significantly higher with overexpression of Ngn3. (e) 36% higher compared to empty vector group in GmGSCs. (f) Similarly, expression of STRA8 in GmGSCs-I-SB was 38% higher. ** $P < 0.01$.

together with NKX2-2. Ngn3 also is required for specification of a common precursor of pancreatic endocrine cells, neurogenesis and spermatogenesis. The other two members of this neurogenin family are neurogenin1 (Ngn1) and neurogenin2 (Ngn2), both of which contain a bHLH domain, and play an important role in determination of sensory lineages in the peripheral nervous system (12,16,17).

In the dairy goat, Ngn3 is expressed widely in the heart, liver, spleen, lungs, kidney, pancreas and testis. This indicates that Ngn3 plays important roles in these tissues, especially in heart and lung, and beyond the nervous system or pancreas, for their high expression levels of Ngn3. It is a marker of differentiating SSCs (5,6) and its expression level increases gradually, as type A_s spermatogonia differentiate. Ngn3 is first expressed at the prepubertal stage in CD117-negative pre-spermatogonia, and is the first reported gene that delineates these earliest stages of spermatogenesis (6,8). In adult goat testis, low ratio of SSCs to all other cell types leads to the apparently lower general expression level of Ngn3, compared to other organs. It also illustrates that low expression level does not mean poor function.

In the pancreas, previous studies have shown that Ngn3 is a marker of early precursors of pancreatic endocrine cells; the Ngn3 knockout mouse cannot generate pancreatic endocrine cells and dies soon after birth (41).

Furthermore, expression of transcription factors Isl1, Pax4, Pax6, NeuroD cannot be detected in them (41,42). Thus, *in vivo*, it is possible that a signal molecule connects Ngn3 with Isl1, Pax4, Pax6, and NeuroD. Besides, HNF-6 (hepatocyte nuclear factor 6) can bind to and stimulate the Ngn3 gene promoter (43).

In the testis, Ngn3 plays a similar role, as a transcription factor, in promoting SSCs to differentiate; however, the germ cell line regulatory network around Ngn3 is little known. A recent study has shown that Ngn3 is a target gene of phosphorylated signal transducer and activator of transcription 3 (STAT3) in progenitor spermatogonia (9). In the process of restraining expression of Ngn3, glial cell line-derived neurotrophic factor (GDNF) plays a key role, but the concrete regulatory mechanism is ambiguous. In goat testis, our results have first shown that Ngn3 promoted expression of Stra8 and CD117 to accelerate the process of spermatogenesis and meiosis, and suppress expression of Plzf (Fig. 8).

Depleted in azoospermia-like (DAZL), an RNA-binding protein and key intrinsic factor, enables spermatogonia to express Stra8 and initiate differentiation and meiosis in response to RA (44). On the other hand, polycomb-group repressive complex 1 (PRC1), chromatin-modifying transcriptional repressors antagonize retinoic acid signalling and maintain Stra8 in a repressive

Figure 8. Summary of the role of Ngn3 in testis. Ngn3 stimulated expression of Stra8 and CD117, and suppressed Plzf; Dazl promotes expression of Stra8; however, PRC1 inhibits Stra8 expression.

chromatin state (45), yet, how the differentiating signal is conveyed to Stra8 after overexpressed Ngn3, is still unknown. Thus, it is worth exploring relevance between Ngn3 and DAZL, or the relationship between Ngn3 and PRC1. In the pancreas, HNF-6 can positively regulate Ngn3; meanwhile, STAT3 directly upregulates expression of Ngn3 in testis. This suggests that there are different specific factors in different tissues being responsible for promoting expression of Ngn3. The role of Ngn3 in regulating spermatogenesis is complex (46,47).

In conclusion, the present study demonstrates that *Ngn3* is a conserved gene, which is highly expressed in adult dairy goat testis. Overexpression *Ngn3* can induce spermatogonia to differentiate, by suppressing self-renewal marker (Plzf) and promoting CD117 and Stra8 to initiate spermatogenesis and meiosis. In addition, Ngn3 can promote expression of Stra8 indirectly. This study further helps us to understand the molecular mechanism of spermatogenesis and meiosis, and can help identify new targets to treat infertility.

Acknowledgements

This work was supported by grants from the Program (31272518) of National Natural Science Foundation of China, Doctoral Fund of Ministry of Education of China (RFDP, 20120204110030), Key Laboratory of the Inner Mongolia Autonomous Region Mammalian Reproductive Biology and Biotechnology of Education Ministry (Inner Mongolia University) Open Major Basic Research Project (20130902), and the Fundamental Research Funds for the Central Universities (QN2011012).

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Supporting Information

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

Fig. S1. HE staining of dairy goat testes in three different age groups. S, Sertoli cell; G, germ cell; D, spermatid. Scale bar = 50 μ m.

Fig. S2. Immunofluorescent staining of Ngn3, Stra8, CD117 and Plzf in GmGSCs and GmGSC-I-SBT. Scale bar = 200 μ m.

Fig. S3. GFP-positive cells in Hela cells, GmGSCs and GmGSCs-I-SB after transfection 24 h in Luciferase Reporter Assay. Scale bar = 200 μ m.