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Expression of goat RNA binding protein (NANOS2), development and evaluation of anti-NANOS2 IgY antibodies

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ABSTRACT: Available rabbit anti-NANOS2 antibodies failed to recognize the NANOS2 protein of goat origin though they showed better reactivity towards the same protein of human and mice origin. A specific antibody against goat NANOS2 is necessary to explore the biological functions of dairy goat NANOS2 protein. Recombinant NANOS2 protein (dairy goat origin) was expressed in *E. coli* (BL21). Anti-NANOS2 immunoglobulin Y (IgY; in chicken) was developed and evaluated. NANOS2 protein sequences between mice and dairy goat were compared using bioinformatics tools. The specific IgY titer attained the peak (≥ 1 : 128 000) after the 3rd booster injection, then it gradually decreased to 1 : 16 000 and remained stable. The specificity and sensitivity of anti-NANOS2 IgY was superior to rabbit anti-NANOS2 IgG. Though, the functional domain 63–116 or 62–115 of NANOS2 protein is highly conserved with 100% identity in dairy goat and mice, the considerable difference in sequence at region 18–61 was identified from the predicted epitopes. According to our survey, this is the first report on preparation of chicken IgY against NANOS2 protein and indicating the prerequisite of species specific NANOS2 antibodies due to epitopes difference in functionally conserved NANOS2 protein. Anti-NANOS2 IgY could be applied as a novel research tool in developmental biology.

Keywords: NANOS2 protein; dairy goat**;** chicken egg yolk antibody (IgY); protein sequence analysis

INTRODUCTION

Germ cells are highly specialized cells for transforming the genetic information from parents to offspring (Suzuki and Saga 2008). The sexual development of mammalian germ cells and their differentiation is a complex and important process governed by different gene regulation systems. Expression of the *Nanos* family of RNA binding proteins by germ cells is evolutionarily conserved in both vertebrates and invertebrates, in which NANOS2 plays an important role in the differentiation of male germ cells by suppression of meiosis (Suzuki and Saga 2008; Orwig and Hermann 2011). Meiotic study is one of the important research areas in the field of developmental biology. Regulations of meiosis have been extensively reported in two mammalian representatives: mouse and humans, but in ruminants, especially in dairy goats, their clear exploration is still missing (Olivier et al. 2005).

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Dairy goat is one of the most extensively distributed domestic animals in the world, the number of dairy goats of the total goat population accounts for 30.9% in developed countries and 19.1% in developing countries (Olivier et al. 2005; Li et al. 2013d). Guanzhong dairy goat (*Capra hircus*) is a milk and meat goat breed originating from the Shaanxi province in northwestern China. It is very popular because of its fast growing and increased reproductive rate. In the recent past, a number of molecular genetic studies in this particular goat variety have been published with noticeable outcomes (Qi et al. 2009; An et al. 2010; Ding et al. 2010). The molecular mechanisms of some genes (*NGN3, PLZF*, *BOULE*, and *MSX1*) in the reproduction of Guanzhong dairy goat were reported previously (Li et al. 2013a; Mu et al. 2013; Song et al. 2013; Tang et al. 2013). Currently, research into the biological functions of NANOS2 RNA binding protein of Guanzhong dairy goat with reference to male germ cell development is the main focus of a part of our research team. One of the most important research tools for the afore-mentioned research focus is anti-NANOS2 antibodies. Unfortunately, the commercial rabbit anti-NANOS2 antibodies showed less affinity towards NANOS2 protein of goat origin even though they reacted well with the same protein of mice and human origin. This prompted the focus of the present study to develop highly specific antibodies against recombinant NANOS2 of dairy goat origin.

Both the development of antibody engineering and animal welfare to reduce the pain of animal paved the way to generate antibodies in laying hens with high yield, convenience, and cost-effectiveness (Xu et al. 2011). The phylogenetic distance between mammals and chickens renders more advantage for the preparation of antibodies in chickens against a wide range of highly conserved mammalian proteins (Kovacs-Nolan and Mine 2004). Hence, the present study was aimed at preparing the chicken immunoglobulin Y (IgY) against recombinant NANOS2 RNA binding protein of dairy goat origin and comparing the similarity and difference between the NANOS2 of mice and dairy goat origin using bioinformatics tools to identify the difference in antigenic determinants. The anti-NANOS2 IgY antibodies could be applied as an efficient research tool in the field of developmental biology as an alternative to mammalian sourced antibodies.

MATERIAL AND METHODS

Preparation of recombinant NANOS2 protein. In order to obtain the NANOS2 protein of Guanzhong dairy goat origin (target gene), the PMD18-T-*NANOS2* vector has been constructed; the target gene was amplified using specific primers (5'-AGCTGTCGCTACCTGACGGTC-3' and 5'-CAGGATCCACTGGTGCCATG-3') by PCR (Cao et al. 2011; Hua et al. 2011; Yao et al. 2014). Amplified clones were flanked with Bam HI and Bgl II sites (double digestion) and the resulted fragment was inserted into the pET28a (+) prokaryotic expression vector. The construction of a proper recombinant DNA fragment was confirmed by DNA sequencing (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China). Then the PET28a-*NANOS2* constructs were transformed into DH5α-competent *Escherichia coli*; then the transformant *E. coli* was grown in Luria-Bertani (LB) broth and the plasmid DNA (recombinant) was isolated using plasmid mini kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China).

In order to express the recombinant NANOS2 protein in *E. coli*, recombinant pET28a constructs were transformed into *E. coli* BL21 (DE3) (Tiangen). Protein preparation of induced cultures (small-scale) was done in a medium containing isopropyl-1-thio-β-D-galactopyranosid (IPTG) with optimization of inducer concentration and induction time. The integrity of recombinant protein expression was screened by the Western blot analysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Hu et al. 2012). Large-scale cultures (500 ml) of the recombinant clones were grown in LB broth with inducer and the cells were harvested by centrifugation. The cells were primarily lysed using a lysis buffer (50 mmol/l Tris-HCl, 1 mmol/l EDTA, pH 8.0, 1mM PMSF) followed by sonication and the supernatant containing the expressed protein was collected by centrifugation and dialyzed. Then they were chromatographed by fast-flow column previously equilibrated with the binding buffer (20mM Tris-HCl, 0.5mM NaCl, 8M urea, 10% glycerinum, pH 7.4) using the AKTA Purifier system (Amersham Pharmacia Biotech, Uppsala, Sweden) with His-tag and eluted with washing buffer (20mM Tris-HCl, 0.5mM NaCl, 8M urea, 10% glycerinum, 500mM imidazole, pH 7.4). The identity, integrity, and purity of the expressed recombinant protein

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were determined by SDS-PAGE and Western blot assay (Zhu et al. 2013).

Sequence alignment between NANOS2 protein of dairy goat and mice. The amino acid sequences were aligned with the known sequences in GeneBank by DNAMAN software (Version 6.0.3.48, 2005) (used for comparison of identity among species). The amino acid sequence of dairy goat NANOS2 was aligned with that of mice NANOS2 in GeneBank by DNASTAR Protean software (Version 5.00, 2001). The epitopes of NANOS2 protein among dairy goat and mice were determined by graphic analysis using the Protean module (DNASTAR). Hydrophilicity, antigenic index, and surface probability were analyzed by methods of Kyte2Doolittle, Emini, and Jameson-Wolf, respectively. Among different species, secondary structures of NANOS2 proteins were predicted by SOPMA website and motifs of NANOS2 protein were analyzed by Gene Runner software (Version 4.0.9 Beta, 2013). Domain of NANOS2 protein of dairy goat was analyzed by NCBICD-Search, Pfam and SMART website, respectively. Predicted 3D model of dairy goat NANOS2 protein was performed by SWISS website. The websites in this test are all listed in Table 1.

Preparation of anti-NANOS2 IgY

Experimental chickens and immunization. The experimental protocol was reviewed and approved by the Ethics Committee of the Northwest Agriculture and Forestry University for the Use of Laboratory Animals. 16-weeks old egg laying hens (2 kg of body weight) were maintained in properly fabricated cages and food and water were offered *ad libitium*. The recombinant NANOS2 protein (1 mg/ml) was emulsified with an equal volume of Freund's complete adjuvant (FCA) for the first immunization and birds were intramuscularly immunized (*Musculus pectoralis*, left and right) with 500 µl of antigen emulsion (NANOS2 protein with adjuvant) at multiple sites. Booster immunization was given 3 weeks after the first immunization in the same manner by replacing FCA with Freund's incomplete adjuvant (FIA). Eggs were collected daily from the first day post-immunization, marked, and stored at 4°C until further processing.

Isolation and purification of anti-NANOS2 IgY. Anti-NANOS2 IgY was extracted and purified using PEG-6000 as described before (Polson et al. 1980). In brief, the yolk of a single egg was separated and diluted 1 : 2 with sterile PBS (0.01M, pH 7.4) with vortex. To eliminate the lipids and lipoprotein, PEG-6000 at a concentration of 3.5% (W/V) was added to the yolk suspension and subjected to gentle shaking at room temperature for 10 min, followed by centrifugation (HC-3018R; Anhui USTC, Zonkia Scientific Instruments Co., Ltd., Beijing, China) at 12 000 *g* , 4°C, for 20 min. The supernatant was filtered and PEG-6000 was added to a final concentration of 12% (W/V) according to the liquid volume. The mixtures were thoroughly stirred and centrifuged at 12 000 *g* for 20 min. The precipitate was dissolved in 10 ml PBS and further precipitated with 12% PEG-6000. Finally, the precipitate was dissolved in 1.2 ml PBS, transferred into a microdialyzer, and dialyzed with PBS overnight at 4°C.

Determination of anti-NANOS2 IgY titres. The indirect ELISA was prepared to determine the specific antibody titer. The prepared recombinant NANOS2 antigen was dissolved in a 0.05 mol/l carbonate buffer (pH 9.6) and used to coat ELISA plates (5.0 µg of 100 µl per well); then the plates were incubated overnight at 4°C. The plates were washed three times with PBS containing 0.5% Tween-20 and blocked for 1 h at 37°C with PBS containing 5% skim milk powder (200 µl per well). Emptied plates were washed three times with PBS containing 0.5% Tween-20, dried, and stored at 4°C. Multiple diluted anti-NANOS2 antibodies in PBS containing 5% skim milk powder (100 µl per well) were added to the ELISA plates, incubated at 37°C for 1 h, washed three times with PBS containing 0.5% Tween-20, and tapped out. Horseradish peroxidase labelled goat anti-chicken IgY (Abcam, Cambridge, UK) diluted at 1 : 6000 in PBS with 5% skim milk powder (100 µl) was added and incubated at 37°C for 1 h. The plates were washed three times with PBS containing 0.5% Tween-20 and dried thoroughly. Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich Shanghai Trading Co., Ltd., Shanghai, China) solution containing 0.1 mmol/l citrate-phosphate buffer and 1 µl/ml H_2O_2 (100 µl) were added to each well and incubated at 37°C for 15 min. 2 mol/l H_2SO_4 (50 µl per well) were added to the ELISA plates for termination. Colour changes were observed and the plates were read directly on a microplate reader at 450 nm (ELx800™; BioTek, Winooski, USA). The titres of anti-NANOS2 IgY were determined by the maximum dilution of the sample (P/N ≥ 2.1, positive control and negative control, 3 replications).

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Characterization of anti-NANOS2 IgY

Western blot analysis. In order to determine the purity and specificity of the anti-NANOS2 IgY, NANOS2 protein was separated by conventional SDS-PAGE and transferred to PVDF membranes, then probed with primary antibodies such as anti-His monoclonal antibody (1 : 1000; Tiangen) and anti-NANOS2 IgY antibody (1 : 100, 1 : 1000, 1 : 2000, 1 : 3000, own preparation). Horseradish peroxidase-conjugated goat anti-mouse IgG (1 : 1000; Beyotime, Haimen, China) or goat anti-chicken IgY (1 : 5000, Abcam) were used as secondary antibody. The results were detected by using the Thermo Scientific™ Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific Inc., Waltham, USA) and analyzed by Tanon-410 automatic gel imaging system (Tanon Science and Technology Co., Ltd., Shanghai, China) (Li et al. 2013c).

Immunohistochemical staining. Testes from 12-month-old dairy goat were fixed, sectioned, and stained for identification of anti-NANOS2 IgY antibody specificity and NANOS2 expression. The tissues were dissected and fixed in 4% paraformaldehyde for 24 h embedded in paraffin wax, sections cut, deparaffinized and rehydrated following standard methods (Li et al. 2013b). Slides were soaked in boiling citrate buffer for 15–25 min, followed by three washes in cold phosphate buffered saline (PBS), each for 5 min. Then the samples were incubated in 3% H_2O_2 for 10 min to inhibit endogenous peroxidase activity and rinsed in PBS. Blocking was performed with 4% goat serum at room temperature for 20 min. Then the slides were exposed to primary antibody (anti-NANOS2, 1 : 100; own preparation) at 4°C overnight or at room temperature for 1–2 h. Goat anti-chicken IgY was used as conjugated secondary antibody (1 : 1000, Abcam), and chromogen solution 3, 3-diaminobenzidine (DAB) was used according to the manufacturer's manual (Beijing Zhongshan Golden Bridge Biochemical Factory, Beijing, China). Concurrently, negative controls were stained with conjugated secondary antibodies alone (Zhu et al. 2012).

Indirect immuno-fluorescence (IIF). GmGSCs-I-SB (immortalized male goat stem cells) were established by transfection of SV40 large T antigen and Bmi1 into primary germ line stem cells and the cell line was maintained as per the standard cell culture procedure (Zhu et al. 2014). The recombination plasmid p*NANOS2*-IRES2-AcGFP1 has been constructed and introduced into the dairy goat testicular cell lines, GmGSCs-I-SB by transfection in 48-well plate or 12-well plate by using Lipofectamine™ 2000 (Invitrogen of Thermo Fisher Scientific Inc., Waltham, USA) according to manufacturer's instructions (Yao et al. 2014). After 4–6 h, conditioned medium was changed to DMEM/F_{12} and cells were further incubated overnight. The GFP positive cells were examined after 24–48 h. The transfected cells were subjected to indirect immunofluorescence. The dairy goat mGSCs-I-SB were fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature, and permeabilized with 0.1% Triton X-100 for 10 min, followed by washing three times with PBS supplemented with 0.1% Tween 20. After being blocked with 1% BSA (Sigma-Aldrich Shanghai Trading Co.) at room temperature for 30 min, cells were incubated in primary antibodies overnight at 4°C. Antibodies were used with the following dilutions: anti-NANOS2 rabbit polyclonal antibody (1 : 300; Santa Cruz Biotechnology, Inc., Dallas, USA) and anti-NANOS2 IgY (1 : 100; own preparation). After three washes in PBS, cells were incubated in 594 anti-rabbit IgG (1 : 500; Bioscience Research Reagents of Merck Millipore, Temecula, USA) and goat anti-chicken IgY (the second antibody; 1 : 1000; Abcam) in the dark at room temperature for 1 h, respectively, followed by three washes in PBS, then the cells, which were incubated in primary anti-NANOS2 IgY, were incubated in Cy3-labelled donkey anti-goat (the third antibody; 1 : 100; Beyotime) in the dark at room temperature for 1 h, then three-times washed in PBS. Finally both cells were incubated in DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich) at room temperature for 1 min. Images were captured and analyzed using a fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) (Liu et al. 2011).

RESULTS

Amplification of NANOS2 protein gene. The DNA fragment with 417 bp in size was amplified from the pMT18-T-*NANOS2* vector (Figure 1a) and identified by BamHI, BglII double restriction enzyme digestion after cloned into the pET28a (+) expression vector. The construction of proper recombinant DNA fragment with target gene was confirmed by DNA sequencing (Sangon Biotech) and uploaded to GeneBank (*NANOS2* cDNA (GenBank accession No. KC155624.1)).

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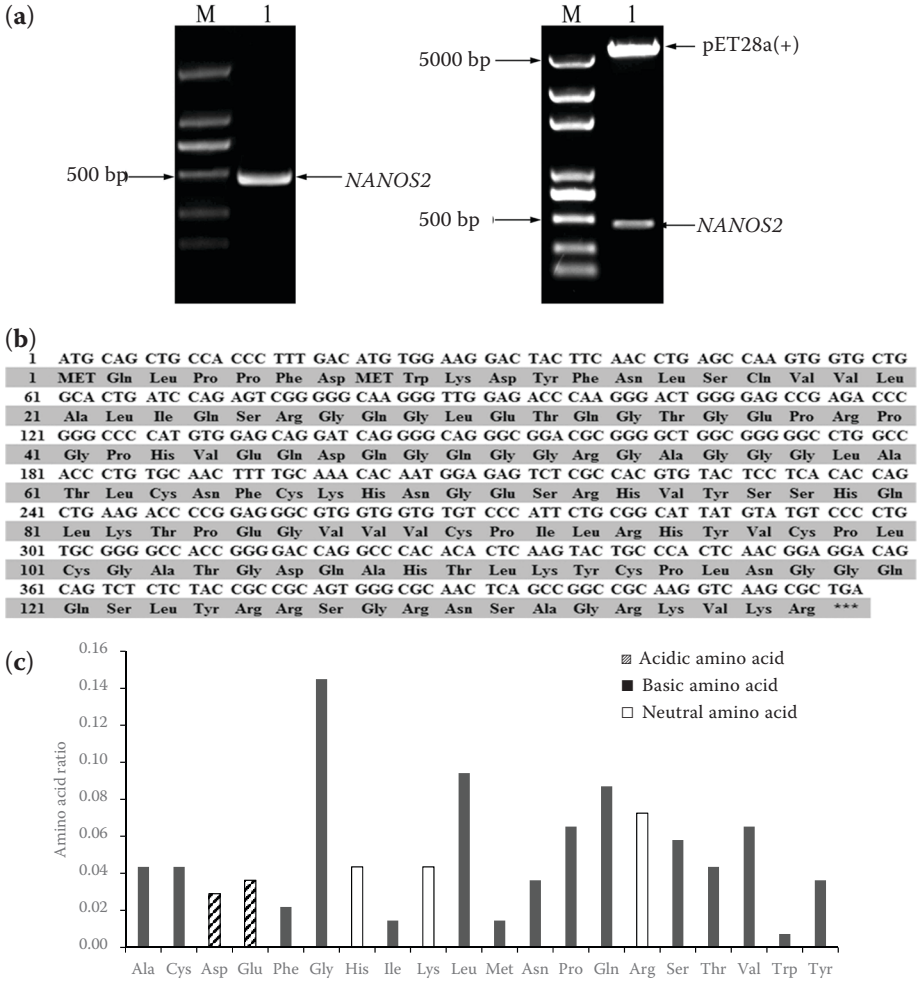

Figure 1. (**a**) Electrophoretogram of PCR product of *NANOS2* gene. A fragment (417 bp) of *NANOS2* was amplified from pMT18-T-*NANOS2* by RT-PCR. (**b**) Restriction enzyme digestion analysis of pET28a-*NANOS2*. (**c**) Nucleotide and deduced amino acid sequences of cDNA encoding NANOS2 protein from Guanzhong dairy goat
***stop codon

Sequence analysis of NANOS2 protein. Primary structure analysis indicated that the molecular weight of the NANOS2 protein of the dairy goat is 15.05 kDa with a theoretical pI = 9.32. The NANOS2 protein containing only 25 charged amino acid residues, which were considered as hydrophilic, exhibited a high content of hydrophobic amino acid residues of Leu (9.3%), Val (6.5%), and Gly (14.5%) (Figure 1b, c). The amino acid sequences of NANOS2 among different species have been aligned by DNAMAN software (Figure 2a). The results revealed that NANOS2 protein is highly conserved in different species with the total identities of 89.04% and the iden-

Table 1. List of databases and websites used for the sequence analysis

Database	Website
NCBI	http://www.ncbi.nlm.nih.gov
SOPMA	http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html
Swiss	http://swissmodel.expasy.org/
NCBI CD-Search	http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
Pfam	http://pfam.janelia.org/search/sequence
SMART	http://smart.embl-heidelberg.de/
ProtScale	http://web.expasy.org/protscale/

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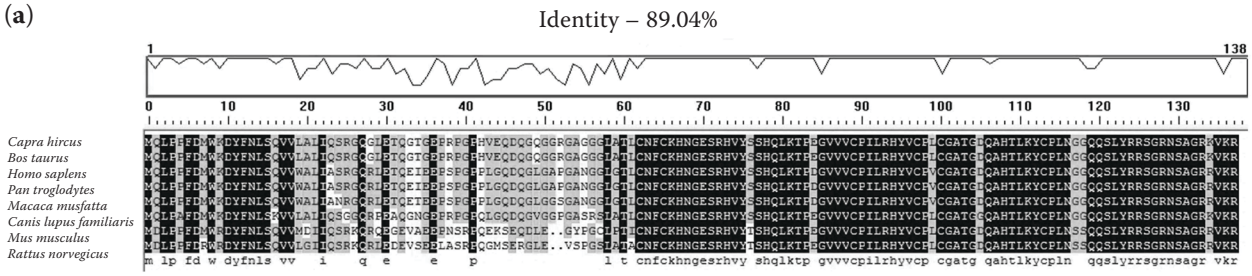
(**a**)



(**b**)

Species	Alpha helix	Extended strand	Beta turn	Random coil
Capra hircus	32	19	8	79
Bos taurus	32	19	8	79
Homo saplens	30	25	6	77
Pan troglodytes	30	25	6	77
Macaca musfatta	32	22	6	80
Canis lupus familiaris	28	16	6	88
Mus musculus	24	23	4	85
Rattus norvegicus	30	19	7	90

(**c**)

Species	Amidation site	N-glycosylation site	Protein kinase C phosphorylation site	N-myristoylation site	Casein kinase II phosporylation site	Tyrosine kinase phospohorylation site
Capra hircus	132	14	110, 127	51, 54, **56**, 57, 118		
Bos taurus	132	14	110, 127	51, 54, **56**, 57, 118		
Homo saplens	132	14	110, 127	**45**, 51, 54, 57, **60**, 118		
Pan troglodytes	132	14	110, 127	**45**, 51, 54, 57, **60**, 118		
Macaca musfatta	132	14	110, 127	**45**, **49**, 51, **52**, 54, 57, **60**, 118		
Canis lupus familiaris	132	14	110, 127	**45**, 51, 54, **102**, **106**, 118		
Mus musculus	**130**	14, **115**	**25, 108. 125**	**55**	**46**	**45**
Rattus norvegicus	**130**	14, **115**	**25, 46, 108. 125**	**21**, **55**		

(**d**)

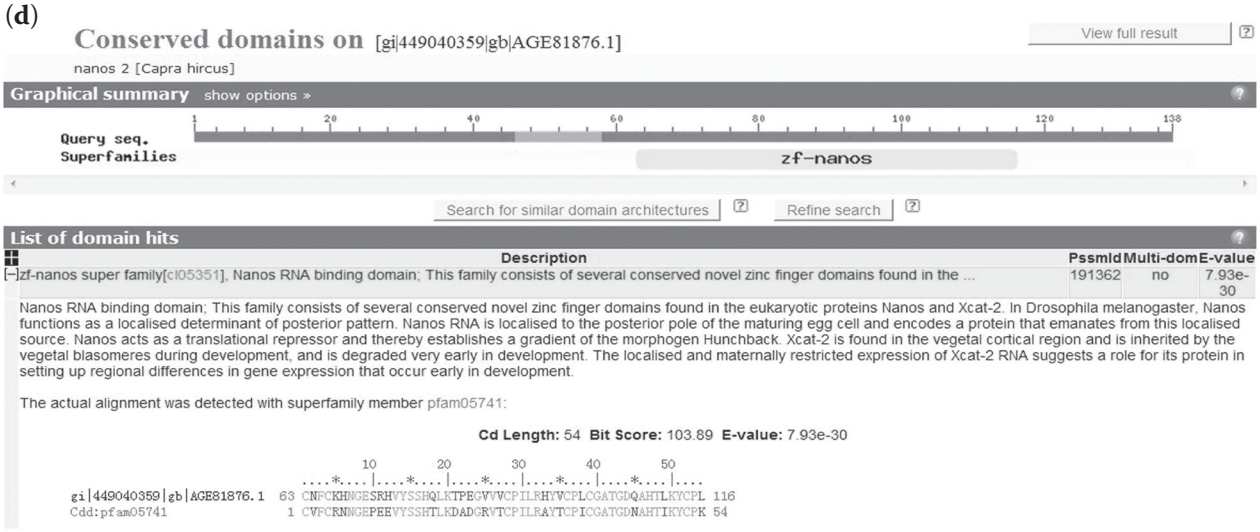


Figure 2. (**a**) Homology comparison of NANOS2 amino acid sequence. (**b**) Secondary structure analysis of NANOS2 protein. (**c**) Motif analysis of NANOS2 protein (non-bolded indicates sites that are highly consistent among mammals). (**d**) Domain analysis of dairy goat NANOS2 protein by NCBI

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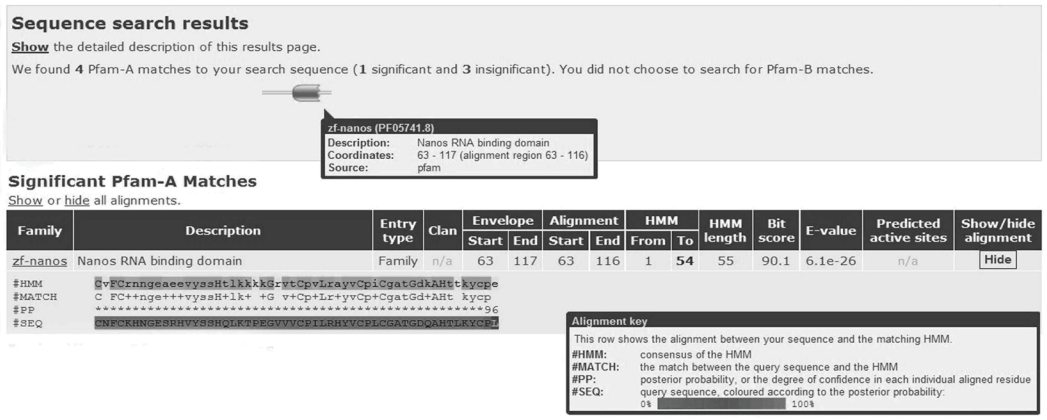
tity of NANOS2 protein between dairy goat and mice of 74.64% (data not shown). The secondary structure of different species was predicted by SOPMA (for www link see Table 1) (Figure 2b). The number of α-helix is 24–32. The extended strand is 16–25, β-turn 4–8. The random coil is 77–85. The amount of components in each kind of protein is similar, which indicated NANOS2 protein is evolutionarily conserved.

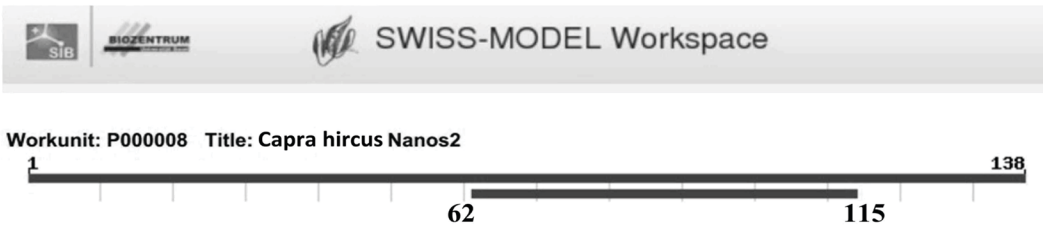
By the motif analysis, we found that some sites of NANOS2 protein are highly conserved among most species (Figure 2c), such as the amidation site, N-glycosylation site, protein kinase C phosphorylation site, and N-myristoylation site. Among the mammals, the sites of motif are conserved except *Mus musculus* and *Rattus norvegicus*, for instance amidation site is basically located at 132 bp in the majority of mammals but in *Mus musculus* and *Rattus norvegicus* it is located at 130 bp. The same dissimilarity was observed in N-glycosylation site, protein kinase C phosphorylation site, and N-myristoylation site. In addition, only *Mus musculus* has casein kinase II phosphorylation site and tyrosine kinase phosphorylation site among aligned species.

As a zinc-finger RNA-binding protein, NANOS has been proposed as an important factor for germline stem cell function (Wang and Lin 2004; Draper et al. 2007). In this study, the amino acid sequence of NANOS2 was subjected to NCBI CD-search to find the typical domain. It was found that the typical NANOS binding domain (zf-nanos) was located at dairy goat NANOS2 protein of 63–116 (Figure 2d). To make the results more reliable, we also carried out a sequence run in Pfam and SMART and obtained the same result that NANOS binding domain

(**a**)



(**b**)



(**c**)

Tool	Predicted functional areas	
Conserved domains	zf-nanos	63-116
SWISS-MODEL	3alrD	62-115
Pfam	zf-nanos	63-116

(**d**)

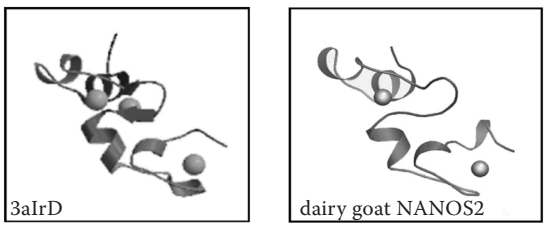


Figure 3. (**a**) Domain analysis of dairy goat NANOS2 protein by Pfam. (**b**) Domain analysis of dairy goat NANOS2 protein by SMART. (**c**) Statistics of predicted functional regions. (**d**) Predicted 3D model of NANOS2 protein

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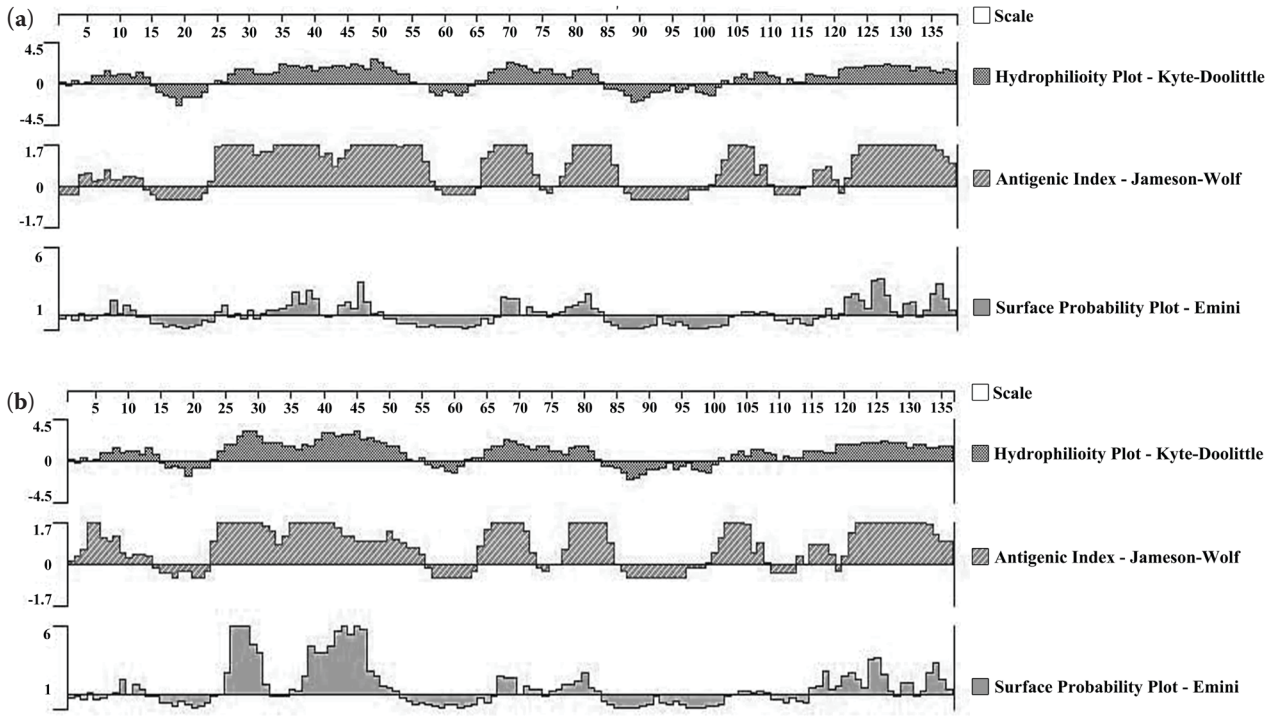

Figure 4. (**a**) Analysis of epitopes in dairy goat NANOS2 protein. (**b**) Analysis of epitopes in mice NANOS2 protein

(zf-nanos and 3aIrD; 3aIrD is the crystal structure of NANOS) is located at dairy goat NANOS2 protein of 63–116 and 62–115, respectively (Figure 3a–c). For further understanding of the relationship between structure and function of NANOS2 protein, modelling of dairy goat NANOS2 protein was performed to predict and estimate its three-dimensional structure. The sequence identity of the dairy goat NANOS2 and known crystal structure protein 3aIrD is 62.96% (Figure 3d).

Prediction of NANOS2 protein epitopes between dairy goat and mice was analyzed by DNASTAR Protean software (Figure 4 a, b). Synthesized analysis including hydrophilicity, antigenic index, and surface probability revealed that antigenic peptides of the dairy goat NANOS2 protein might be 33–48, and that of mice might be 25–51, and indicated a huge difference between dairy goat and mice in NANOS2 protein sequence.

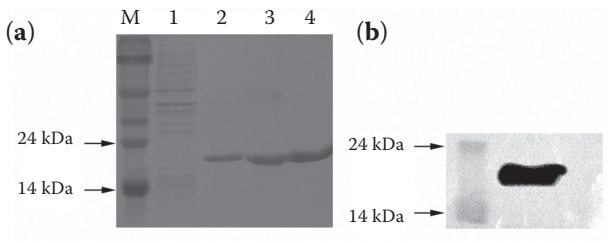

Figure 5. (**a**) Purification and identification of recombinant fusion proteins with SDS-PAGE. M = standard maker, 1 = induced pET28a(+), 2–4 = three times purified fusion proteins. (**b**) Western blot analysis of recombinant protein with His-tag

Purification of His-tagged recombinant NANOS2 protein. The DNA fragment of *NANOS2* gene was inserted into pET28a (+) vector for protein expression. The pET28a (+) vector is designed for inducible and high level expression of *NANOS2* gene fragment as fusion protein with a His-tag. Target protein including the His-tag was expressed with molecular weight of 15.87 kD after a 6-hour induction by 1 mmol/l IPTG and identified by 15% SDS-PAGE (data not shown). The recombinant NANOS2 protein was purified by AKTA Purifier system with the His-tag and we selected one sample out of each purification to confirm the purified protein by 15% SDS-PAGE (Figure 5a). To further verify the recombinant NANOS2 protein, the Western blot analysis was performed with the monoclonal antibody against the His-tag. The result showed that the recombinant NANOS2 protein fused with His-tag strongly reacted with the monoclonal antibody against His-tag, and the specific reaction band was found to be about 15 kDa in size (Figure 5b).

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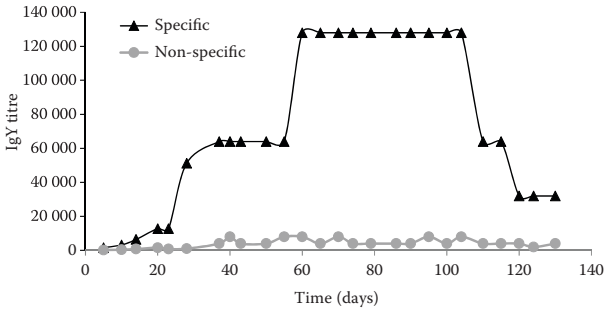

Figure 6. Titre of anti-NANOS2 IgY determined by ELISA

Titre of anti-NANOS2 IgY determined by ELISA. The titre level of specific IgY against recombinant NANOS2 protein in immune egg yolk was determined by iELISA. The specific IgY titre (≥ 1 : 1280) was increased after the first booster injection and attained the peak (≥ 1 : 128 000) after the third booster injection and it was stable at the highest titre for more than one month. Then it was gradually decreased to 1 : 16 000 and remained at the same level till the end of the experiment (Figure 6).

Characterization of anti-NANOS2 IgY. Crude recombinant NANOS2 protein was subjected to Western blotting in order to evaluate the optimal dilution ratio of anti-NANOS2 IgY. It was found that the target protein could be clearly detected even when the dilution ratio was 1 : 3000 (Figure 7). Further, the specificity of anti-NANOS2 IgY antibody was assessed by immunohistochemical staining. The result indicated that the NANOS2 protein was expressed in adult dairy goat testis (Figure 8). This finding was in accordance with the previous research done on mice reported by Susuzki et al. 2009. The dairy goat mGSCs-I-SB was transfected with recombinant plasmid p*NANOS2*-IRES2-AcGFP1 as well as with empty vector pIRES2-AcGFP1. Both the groups have had GFP positive cells for 24 h after transfection, which indicated that the transfection process was successful. Then the efficiency of anti-NANOS2 IgY antibody was compared with rabbit polyclonal anti-NANOS2 antibody by immunofluorescence assay. The result revealed that when anti-NANOS2 IgY antibody was used as the primary antibody, the red fluorescence could be detected highly in mGSCs-I-SB cells with over expression of NANOS2 protein when compared to the rabbit anti-NANOS2 antibody (Figure 9). The assessment method demonstrated that anti-NANOS2 IgY antibody possesses excellent specificity and sensitivity.

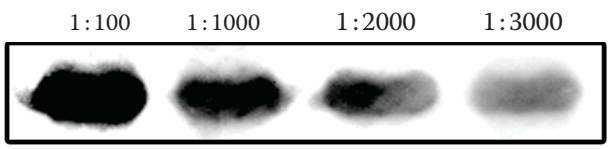

Figure 7. Identification of anti-NANOS2 IgY antibody dilution ratio by Western blot analysis

DISCUSSION

The RNA binding protein NANOS2 containing a zinc finger motif plays a significant role in male reproduction and also maintains the self-renewal of spermatogonial stem cells (SSCs) (Olivier et al. 2005). Molecular mechanism of the NANOS2 protein is extensively studied in mice and humans; to understand the biological function of NANOS2 RNA binding protein of Gunazhong dairy goat, an attempt was made by our research group, unfortunately the commercially available rabbit anti-NANOS2 antibodies did not satisfactorily recognize the NANOS2 of goat origin though it showed better affinity towards the same protein of human and mice origin. This finding formed the way to fix the goal of this study to prepare specific antibody against NANOS2. Immunoglobulin Y (IgY) isolated from the eggs of immunized chickens has several attractive advantages over conventional mammalian IgG. In particular remarkable affinity and avidity of IgY antibodies against the highly conserved mammalian proteins is an important advantage due to evolution divergence of avian species (Larsson et al. 1993).

In this study, *NANOS2* gene fragment of 417 bp was amplified using the pMD18-T-*NANOS2* (constructed and stored in a laboratory) in order to express the Guanzhong dairy goat NANOS2 protein (138 amino acids with molecular weight of 15.05 kD). The prokaryotic expression system was employed and then the purity and specificity of the recombi-

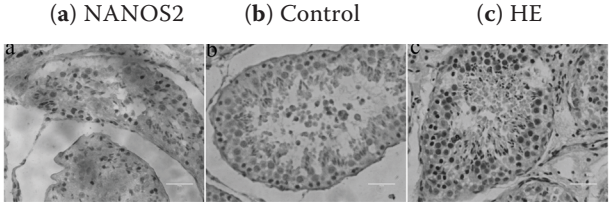

Figure 8. (**a**) Anti-NANOS2 IgY antibody immunohistochemical staining of adult dairy goat testicular tissues. (**b**) Control group for immunohistochemical staining of adult dairy goat. (**c**) HE staining of the adult dairy goat scale bar = 50 μm

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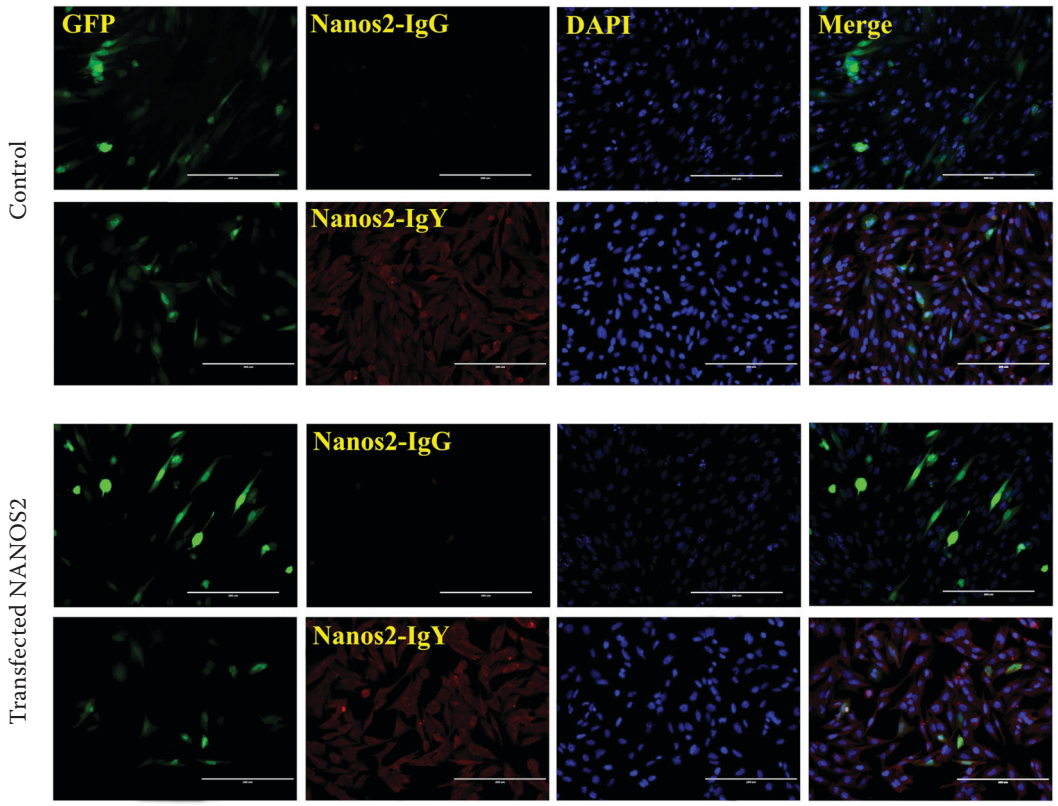

Figure 9. Assessment of anti-NANOS2 IgY antibody by immunofluorescent staining of GmGSCsI-SB after over-expression of *NANOS2*. After *NANOS2* over-expression or not, GmGSCs-I-SB was immunostained with different antibodies including Rabbit polyclonal anti-NANOS2 IgG antibody and anti-NANOS2 IgY antibody (red). GFP expression indicated GmGSCs-I-SB was transfected successfully with p*NANOS2*-IRES2-AcGFP1 and pIRES2-AcGFP1. DNA was labelled with DAPI (blue) (scale bar = 200 μm)

nant NANOS2 was observed using the Western blot (Hardin et al. 2001; Sambrook et al. 2001).

The primary structure, secondary structure, and the motif of NANOS2 protein among species were analyzed, it was found that dairy goat NANOS2 protein was usually conserved and has some similarities with other species. The NANOS binding domain of dairy goat NANOS2 protein and its three-dimensional structure were predicted through three protein analysis sites (Swiss, NCBI CD-Search, and Pfam). The results provided a useful resource for the functional research of Guanzhong dairy goat NANOS2 protein. Protean is a widely used and helpful software for analyzing protein (Konno et al. 2009). In order to find out the reason why the rabbit anti-NANOS2 antibodies failed to recognize NANOS2 of goat origin, the appropriate epitopes of NANOS2 protein of dairy goat and mice have been synthesized and the hydrophilicity, antigenic index, and surface probability were assessed using Protean. Results showed that the region 33–48 of dairy goat NANOS2 protein has comprehensive high index in hydrophilicity and antigen index whereas in mice it exists at region 25–51. Even though, the functional domain 63–116 or 62–115 of NANOS2 protein is a highly conservative sequence with a 100% identity in both dairy goat and mice, the considerable difference in sequence identity at region 18–61 was identified from the predicted epitopes. These results suggested that the function of NANOS2 protein in male reproduction among different species was highly conserved, but the low identity of epitopes made the rabbit anti-NANOS2 antibodies unsuccessful to recognize NANOS2 of goat origin. This result is strongly suggesting the need of specific antibody against NANOS2 protein of goat origin to explore its biological functions in the field of developmental biology. It is also indicating the importance to raise specific antibodies against species specific proteins to have better results while investigating their biological functions.

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It is a well-known concept that the immune response is more effective when the evolutionary distance between the source of antigen and immune system is increased. Therefore, the chickens are always better choice to prepare the immunoreagents with high titer of specific antibody against highly conserved mammalian antigens with high yield because a chicken usually lays 280 eggs/year and an egg yolk (12–15 ml) normally contains 150–200 mg of IgY, of which 2–10% can against target antigen specifically (Schade and Hlinak 1995).

In order to evaluate the specificity and sensitivity of generated anti-NANOS2 IgY, homologous assays such as SDS-PAGE, iELISA, Western blotting, immunohistochemical staining, and immunofluorescence were carried out. The results indicated that anti-NANOS2 IgY antibody showed the better affinity and specific reactivity to the *r*NANOS2 protein of dairy goat origin than anti-NANOS2 antibodies got from rabbit. It is extensively reported that chicken antibodies could bind to more epitopes on a mammalian protein than the corresponding mammalian antibodies and the absence of immunological cross-reactivity between chicken IgY and mammalian IgG was determined by the evolution divergence (Hadge and Ambrosius 1984; Kovacs-Nolan and Mine 2004; Dias da Silva and Tambourgi 2010). These findings strongly support the application of anti-NANOS2 IgY as a research tool to explore the biological functions of NANOS2 protein in male germ cell development.

CONCLUSION

This study reported the preparation of anti-NANOS2 IgY using egg laying chickens and found its reactivity was superior to the commercially available rabbit anti-NANOS2 antibodies due to the difference in antigenic determinants (epitopes) of evolutionary conserved NANOS2 protein. According to our survey, this is the first report on preparation of chicken IgY against NANOS2 RNA binding protein. Importantly, the current study addressed the long lasting research need, i.e., prerequisite of highly specific antibodies against NANOS2 protein of goat origin to explore its biological function in male germ cell development of dairy goat. By considering the advantages of chicken IgY over mammalian IgG for its high yield, convenience and cost-effectiveness with increased affinity, this investigation formed a platform for various application of IgY in the field of developmental biology.

REFERENCES

An X., Hou J., Wang L., Li G., Wang J., Song Y., Zhou G., Han D., Ling L., Cao B. (2010): Novel polymorphisms of the growth hormone gene and their effect on growth traits in Chinese goats. Meat Science, 86, 758–763.

Cao H., Chu Y., Zhu H., Sun J., Pu Y., Gao Z., Yang C., Peng S., Dou Z., Hua J. (2011): Characterization of immortalized mesenchymal stem cells derived from foetal porcine pancreas. Cell Proliferation, 44, 19–32.

Dias da Silva W., Tambourgi D.V. (2010): IgY: a promising antibody for use in immunodiagnostics and in immunotherapy. Veterinary Immunology and Immunopathology, 135, 173–180.

Ding W., Kou L., Cao B., Wei Y. (2010): Meat quality parameters of descendants by grading hybridization of Boer goat and Guanzhong Dairy goat. Meat Science, 84, 323–328.

Draper B.W., McCallum C.M., Moens C.B. (2007): Nanos1 is required to maintain oocyte production in adult zebrafish. Developmental Biology, 305, 589–598.

Hadge D., Ambrosius H. (1984): Evolution of low molecular weight immunoglobulins–IV. IgY-like immunoglobulins of birds, reptiles and amphibians, precursors of mammalian IgA. Molecular Immunology, 21, 699–707.

Hardin C.C., Pinczes J., Riell A., Presutti D., Miller W., Robertson D. (eds) (2001): Cloning, Gene Expression, and Protein Purification. Oxford University Press, New York, USA.

Hu P., Guan W., Li X., Ma Y. (2012): Construction of recombinant proteins for reprogramming of endangered Luxi cattle fibroblast cells. Molecular Biology Reports, 39, 7175–7182.

Hua J., Zhu H., Pan S., Liu C., Sun J., Ma X., Dong W., Liu W., Li W. (2011): Pluripotent male germline stem cells from goat fetal testis and their survival in mouse testis. Cellular Reprogramming, 13, 133–144.

Konno A.Y., Maricato J.T., Konno F.T., Mariano M., Lopes J.D. (2009): Peptides from *Paracoccidioides brasiliensis* GP43 inhibit macrophage functions and inflammatory response. Microbes and Infection, 11, 92–99.

Kovacs-Nolan J., Mine Y. (2004): Avian egg antibodies: basic and potential applications. Avian and Poultry Biology Reviews, 15, 25–46.

Larsson A., Balow R.M., Lindahl T.L., Forsberg P.O. (1993): Chicken antibodies: taking advantage of evolution – a review. Poultry Science, 72, 1807–1812.

Li M., Liu C., Zhu H., Sun J., Yu M., Niu Z., Liu W., Peng S., Hua J. (2013a): Expression pattern of Boule in dairy

doi: 10.17221/8129-CJAS

goat testis and its function in promoting the meiosis in male germline stem cells (mGSCs). Journal of Cellular Biochemistry, 114, 294–302.

Li M., Yu M., Liu C., Zhu H., He X., Peng S., Hua J. (2013b): miR-34c works downstream of p53 leading to dairy goat male germline stem-cell (mGSCs) apoptosis. Cell Proliferation, 46, 223–231.

Li M., Yu M., Liu C., Zhu H., Hua J. (2013c): Expression of miR-34c in response to overexpression of Boule and Stra8 in dairy goat male germ line stem cells (mGSCs). Cell Biochemistry and Function, 31, 281–288.

Li M., Yu M., Zhu H., Song W., Hua J. (2013d): The effects of Nanos2 on Boule and Stra8 in male germline stem cells (mGSCs). Molecular Biology Reports, 40, 4383–4389.

Liu C., Zhu H., Liu W., Zheng W., Wang J., Yang C., Hua J. (2011): Male germ cells specification of embryonic gonad from Guanzhong dairy goat. Chinese Journal of Animal and Veterinary Sciences, 42, 1328–1336.

Mu H., Wu J., Zhu H., Li N., Tang F., Yao X., Yang C., Peng S., Li G., Hua J. (2013): The function of *Msx1* gene in promoting meiosis of dairy goat male germline stem cells (mGSCs). Cell Biochemistry and Function, 31, 629–635.

Olivier J., Cloete S., Schoeman S., Muller C. (2005): Performance testing and recording in meat and dairy goats. Small Ruminant Research, 60, 83–93.

Orwig K.E., Hermann B.P. (eds) (2011): Male Germline Stem Cells: Development and Regenerative Potential. Humana Press, London, UK.

Polson A., VonWechmar M.B. (1980): Isolation of viral IgY antibodies from yolk of immunized hens. Immunological Communications, 9, 475–493.

Qi Y., Luo J., Han X., Zhu Y., Chen C., Liu J., Sheng H. (2009): Genetic diversity and relationships of 10 Chinese goat breeds in the Middle and Western China. Small Ruminant Research, 82, 88–93.

Sambrook J., Russell D.W. (2001): Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York, USA.

Schade R., Hlinak A. (1995): Egg yolk antibodies, state of the art and future prospects. Altex, 13, 5–9.

Song W., Zhu H., Li M., Li N., Wu J., Mu H., Yao X., Han W., Liu W., Hua J. (2013): Promyelocytic leukaemia zinc finger maintains self-renewal of male germline stem cells (mGSCs) and its expression pattern in dairy goat testis. Cell Proliferation, 46, 457–468.

Suzuki A., Saga Y. (2008): Nanos2 suppresses meiosis and promotes male germ cell differentiation. Genes and Development, 22, 430–435.

Suzuki H., Sada A., Yoshida S., Saga Y. (2009): The heterogeneity of spermatogonia is revealed by their topology and expression of marker proteins including the germ cell-specific proteins Nanos2 and Nanos3. Developmental Biology, 336, 222–231.

Tang F., Yao X., Zhu H., Mu H., Niu Z., Yu M., Yang C., Peng S., Li G., Hua J. (2013): Expression pattern of Ngn3 in dairy goat testis and its function in promoting meiosis by upregulating Stra8. Cell Proliferation, 47, 38–47.

Wang Z., Lin H. (2004): Nanos maintains germline stem cell self-renewal by preventing differentiation. Science, 303, 2016–2019.

Xu Y., Li X., Jin L., Zhen Y., Lu Y., Li S., You J., Wang L. (2011): Application of chicken egg yolk immunoglobulins in the control of terrestrial and aquatic animal diseases: a review. Biotechnology Advances, 29, 860–868.

Yao X., Tang F., Yu M., Zhu H., Chu Z., Li M., Liu W., Hua J., Peng S. (2014): Expression profile of *Nanos2* gene in dairy goat and its inhibitory effect on Stra8 during meiosis. Cell Proliferation, 47, 396–405.

Zhu H., Liu C., Li M., Sun J., Song W., Hua J. (2012): Optimization of the conditions of isolation and culture of dairy goat male germline stem cells (mGSC). Animal Reproduction Science, 137, 45–52.

Zhu H., Ma J., Du R., Wu J., Song W., Niu Z., He X., Du E., Zhao S., Hua J. (2014): Characterization of immortalized dairy goat male germline stem cells (mGSCs). Journal of Cellular Biochemistry, 115, 1549–1560.

Zhu Q., Guo D., Feng L., Sun D. (2013): Expression and purification of the scFv from hybridoma cells secreting a monoclonal antibody against S protein of PEDV. Monoclonal Antibodies in Immunodiagnosis and Immunotherapy, 32, 41–46.

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