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Expression Pattern of Boule in Dairy Goat Testis and Its Function in Promoting the Meiosis in Male Germline Stem Cells (mGSCs)

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ABSTRACT

Boule is a conserved gene in meiosis, which encodes RNA binding protein required for spermatocyte meiosis. Deletion of Boule was found to block meiosis in spermatogenesis, which contributes to infertility. Up to date, the expression and function of Boule in the goat testis are not known. The objectives of this study were to investigate the expression pattern of Boule in dairy goat testis and their function in male germline stem cells (mGSCs). The results first revealed that the expression level of Boule in adult testes was significantly higher than younger and immature goats, and azoospermia and male intersex testis. Over-expression of Boule promoted the expression of meiosis-related genes in dairy goat mGSCs. The expression of Stra8 was up-regulated by over-expression of Boule analyzed by Western blotting and Luciferase reporter assay. While, Cdc25a, the downstream regulator of Boule, was found not to affect the expression of Stra8, and our data illustrated that Cdc25a did not regulate meiosis via Stra8. The expression of Stra8 and Boule was up-regulated by RA induction. Taken together, results suggest the Boule plays an important role in dairy goat spermatogenesis and that over-expression of Boule may promote spermatogenesis and meiosis in dairy goat. J. Cell. Biochem. 114: 294–302, 2013.

KEY WORDS: BOULE; MEIOSIS; SPERMATOGENESIS; MALE GERMLINE STEM CELLS (mGSCs); DAIRY GOAT

The mammalian spermatogenesis undergoes proliferation of spermatogonia A; differentiation of spermatogonia B, and further into spermatocyte; formation of round spermatid through meiosis and in the end develop into mature sperm [Houston and King, 2000]. The meiosis of germ cells is a critical step for spermatogenesis. Meiosis related gene missing or mutation frequently leads to azoospermia or male infertility [Yen, 2004]. As a conserved gene in meiosis, Boule (Boll) encodes RNA binding protein, which is required for spermatocyte meiosis. Boule expression is specifically up-regulated during the first meiosis of spermatogenesis (prometaphase) and it is not detectable in meiosis-blocked testis. Boule belongs to Daz family, are found in primates [Xu et al., 2003] and homologue Dazl (deleted in azoospermia-like) in human [Tschanter et al., 2004], mice [Cooke et al., 1996], fish [Xu et al., 2009], zebrafish [Maegawa et al., 1999], vertebrate [Xu et al., 2007], Drosophilae [Eberhart et al., 1996] and *C. elegans* [Karashima et al., 2000]. Boule is an ancestral gene of Daz family, with the characteristic RNA recognition motif in mammals [Houston et al.,

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1998; Shah et al., 2010]. The function of Boule in mammalian is still unclear, however, since it plays an indispensable role in spermatogenesis [Michael et al., 2010]. Lower-expression of Boule resulted in the block of meiosis and spermatogenesis and then azoospermia [Lin et al., 2005]. Dazl functions in primordial germ cell (PGC) formation, whereas its closely related genes Daz and Boule promote later stages of meiosis and development of haploid gametes [Kee et al., 2009; VanGompel and Xu, 2010]. Michael et al. [2010] discovered that meiosis completed normally in the absence of Boule, and haploid round spermatids were readily detected. However, round spermatid did not progress beyond step 6, revealing a novel role for Boule in spermatogenesis, the differentiation of round spermatids into mature spermatozoa [Michael et al., 2010]. Expression of key regulators of spermatogenesis was unaffected in Boule$^{-/-}$ mice, suggesting that Boule regulates germ cell differentiation through a novel pathway [Michael et al., 2010; VanGompel and Xu, 2011]. Previous studies have shown that Boule regulates the expression of Cdc25a, further phosphorylate Cdc2, then activate maturation-promoting factor (MPF), initiate the meiosis [VanGompel and Xu, 2011]. However, the molecular mechanism on how meiosis initiation is very unclear, especially for livestock.

Boule homologue Daz is specifically expressed in Drosophilaee testis [Eberhart et al., 1996]. Xu et al. [2001] identified a Daz/Dazl like protein when they performed yeast hybridization assay to screen Daz/Dazl interaction protein. Through amino acid alignments, they found that the protein shared 42% homologue with Drosophilae, thus named it Boule [Xu et al., 2001; Li et al., 2007]. Boule is responsible for sperm production and appears to be the only gene exclusively required for sperm production from insect to mammal [Shah et al., 2010]. The expression of Boule is critical for spermatogenesis [Shah et al., 2010].

Stra8 (stimulated by retinoic acid 8), a target gene of retinoic acid (RA) signaling, was expressed sex-specifically in female fetal gonads at the time of female-specific meiotic entry [Oulad-Abdelghani et al., 1996; Koubova et al., 2006]. The Cyp26b1 inhibitor of retinoic acid signaling was expressed sex-specifically in male fetal gonads at the same time [Bowles et al., 2006]. Recent studies demonstrated that Stra8 is required for meiotic entry in both male and female germ cells, putting to the hypothesis that the mitosis/meiosis decision might be equivalent to the sperm/oocyte decision. RA is capable of driving both female and male fetal germ cells into meiosis [Kimble, 2011].

Up to date, most reports on Boule were mouse, human, Drosophila, and *C. elegans* [Eberhart et al., 1996; Karashima et al., 2000]. No report on the expression of Boule was obtained in dairy goat. Dairy goat is one kind of domestic animals of great importance not only for its easily absorbed milk and wonderful meat, but also for its shorter gestation length, earlier sexual maturation, and smaller size than cattle for studying lactation, ruminant nutrition, and fetal development. Male GSCs derived from dairy goat testis are valuable resource for the genetic material conservation and optimization [Hua et al., 2011; Zhu et al., 2012]. Here, we investigated to explore the expression pattern of Boule and study its effects on the meiosis of dairy goat mGSCs.

MATERIALS AND METHODS

COLLECTION OF DAIRY GOAT TESTIS

Guanzhong dairy goat testis was supplied by Yaoan slaughterhouse in Yangling Hi-tech area. Testes were collected from the dairy goats at different ages. The goats were killed specifically for our experiment and teaching practice base of Northwest A&F University and Shaanxi Centre of Stem Cells Engineering & Technology; Northwest A&F University approved all the procedures.

IMMUNOFLUORESCENCE STAINING

Dairy goats testicular tissue derived from 106 to 122 days post coitum (dpc), 30 days postnatal (dpp), adult and adult azoospermia were dissected, fixed in 4% paraformaldehyde, respectively. The testicular tissues were paraffined, deparaffinization and rehydration following the standard methods. Slides were dipped in three changes of xylene for 6 min each, two changes of 100%, 95%, 75% alcohol for 3 min respectively, afterwards rinsed twice in deionized water for 5 min. The slides were soaked in the boiling citrate buffer for 15–25 min, followed by three washes in cold PBS, each for 5 min. Washed slides were blocked with blocking solution (PBST + 1% BSA) for a minimum of 30 min and incubated with primary antibody to Boule (1:100, Santa Cruz Biotechnology Inc., CA) overnight at 4°C, Tissues were washed in PBS for three times, and then incubated with secondary antibody (1:500, Chemicon International, Inc., Temecula, CA) following the manufacturer's manual. The nuclei of cells were stained by Hoechst 33342 [Liu et al., 2011].

RT-PCR AND QUANTITATIVE RT-PCR ANALYSIS

Total RNAs for RT-PCR analysis were extracted from different tissues of adult dairy goat and testicular tissues at 90 dpc, neonatal, 25 dpp, 90 dpp, adult, adult azoospermia and male intersex cultured mGSCs using TRIzol (Tiangen Biotech Co. Ltd., Beijing, China). The cDNA was synthesized based on 500 ng RNA with a commercially available kit (TaKaRa, Biotech. Co. Ltd., Dalian, China). The PCR steps included denaturation at 94°C for 5 min, followed-by 35 cycles at 95°C for 30 s, 55–58°C for 30 s, and 72°C for 30–60 s, and a final extension at 72°C for an additional 10 min. The primers were designed based on the sequences of the open reading frame from the NCBI GenBank and synthesized by AuGCT Biotechnology, Beijing. The PCR primers and the lengths of the amplicons are shown (Table I). The PCR products were analyzed in 2% agarose (Invitrogen, Carlsbad, CA) gel electrophoresis, stained with ethidium bromide

TABLE I. The Primer Sequences

Name	Sense primer	Antisense primer
β-actin	5′-acggcatcaccaact-3′	5′-aggaaggaaggctggaagag-3′
Stra8	5′-cggccatctcccatctgtg-3′	5′-tgtccttcacgctgccctc-3′
Scp3	5′-gtatggaggacttggaga-3′	5′-gagactttcggacacttgc-3′
Dazl	5′-caagttcaccagttcaggttatcac-3′	5′-gacaacggagtttctcagtctattc-3′
Cdc25a	5′-acctctttcataccgttgctgg-3′	5′-aactcccttgtatccgcccttca-3′
Cdc2	5′-tctatccctcctggtcagttc-3′	5′-tgtccactggagttgagtagc-3′
Cyp26b1	5′-aaggagatgaccatgcaggag-3′	5′-gcagctgcatgataagcgagg-3′
Oct4	5′-aggagtcccaggacatcaa-3′	5′-agggtgatcctcttctgctt-3′
C-myc	5′-ctggtgggcgagatcatca-3′	5′-cactgccatgaatgatgttcc-3′
Vasa	5′-gctggcgtaatagcgaagagg-3′	5′-gcacagatgcgtaaggagaaaa-3′
CD90	5′-gatccaggactgagctctcgg-3′	5′-tcacgggtcagactgaactcatac-3′

(Invitrogen), and visualized under UV illumination [Cao et al., 2011; Hua et al., 2011].

Quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) was set up in 15 μl reaction mixtures containing dpp H_2O 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: 94°C for 5 min, and then 40 cycles 94°C for 20 s, 58°C for 30 s, and 70°C for 10 s. All expression levels were normalized to β-actin in each well. The double ΔCt method was used to measure the expression alteration [Hu et al., 2012]. The fluorescence signal was collected every 0.5°C for 10 s. The primers are shown in Table I.

CONSTRUCTION OF RECOMBINATION PLASMID

The dairy goat Boule and Cdc25A primers were designed according to the reported *B. taurus* b-Boule cDNA sequence (NM_001102115) and *B. taurus* Cdc25A cDNA sequence (BC_151493). Boule is 1091 base pair (bp) and Cdc25a is 1,684 bp. Primers were synthesized by Peking SanBoYuanZhi Company, Beijing:

Boule: Forward: 5′-GAAAGTCTCCGTCGGGAAGCGT-3′;
Reverse: 5′-GGCAGCTTCTAGCCGGTTCATTG-3′.
Cdc25a: Forward: 5′-AAGATCTAGAGGGAGCGGGATCCCTAGGAG -3′;
Reverse: 5′-AGTCGACAGCAAAGAGGGGAGTGGGGAG-3′.

The genes were amplified from dairy goat testis using RT-PCR. Then the specific fragments were cloned into pMD18-T cloning vector (TaKaRa). After white–blue screening, the positive clones were identified by PCR, restriction enzyme digestion and sequencing. Then the fragments were cloned into pIRES2-EGFP eukaryotic expression vector to obtain pBoule-IRES2-GFP and pCdc25a-IRES2-GFP recombination plasmid respectively. The plasmid was verified by PCR and restriction enzyme digestion.

EVOLUTIONARY RELATIONSHIP AMONG THE BOULE CDS

The Guanzhong dairy goat Boule CDS was obtained, sequenced by Peking SanBoYuanZhi Company, Beijing. Then the sequence was aligned with the known sequences in Genebank by DNAman software and the phylogenetic tree was depicted with MEGA4.1.

CELL TRANSFECTION AND RA INDUCTION

The dairy goat mGSCs were cultured in our lab and previous studies [Ko et al., 2009, 2010; Hua et al., 2011; Zhu et al., 2012]. The pBoule-IRES2-GFP recombination plasmid and pIRES2-EGFP were transfected into dairy goat mGSCs in a 48-well plate. The plasmid DNA was diluted to 200 ng in 50 μl Opti-MEM (Invitrogen) reduced serum medium. Mixed gently, then added 0.5 μl PLUS™ Reagent (Invitrogen) directly to the diluted DNA and incubated the mixed medium for 5 min at room temperature. Mixed Lipofectamine™ LTX Reagent (Invitrogen) gently before using then added 1 μl directly to the diluted DNA. Mixed gently and incubated for 30 min at room temperature. The 50 μl DNA–Lipofectamine™ LTX complexes were added, and incubated the cells for 4–6 h at 37°C in a CO_2 incubator. The GFP positive cells were examined after 48 h, and the total RNAs between two groups were extracted and the germ cell and meiosis markers:Vasa, Stra8 and Scp3 were analyzed by RT-PCR and QRT-PCR [Hu et al., 2012].

To investigate whether the Boule was regulated by RA, transfected Boule mGSCs were treated by 1×10^{-5}, and 1×10^{-6} M RA (Sigma Chemical Co., St. Louis, MO) respectively, and the mRNAs of treated cells were analyzed by QRT-PCR.

LUCIFERASE REPORTER ASSAY

The pStra8-LUC, the 1.4 kb fragment of Stra8 promoter was inserted into SacI/HindIII sites of the pGL3-basic vector (Clontech Laboratories, Inc., CA) [Jia et al., 2011]; 50 ng of pStra8-LUC, pBoule-IRES2-GFP or pIRES2-GFP (negative control) was co-transfected into HeLa cells in a 48-well plate using Lipofectamine2000 (Invitrogen). After 48 h, the target validation assays were performed with luciferase reporter system (Beyotime, Jiangsu, China) according to the manufacturer's instructions.

DUAL-LUCIFERASE® REPORTER ASSAY

The pmiR-RB-Report™-Stra8 3′UTR construct was generated by cloning the entire 3′UTR of Stra8 into pmiR-RB-Report™ vector (Ruibo, Guangzhou, China) at the site which was digested by *Not*I and *Xho*I enzyme. The primers for Stra8 3′UTR: Sence, 5′-TTACTCGAGAGCAGGAGGGCCGGAGA-3′, Reverse, 5′-CGTGCGG-CCGCATTGCTCTTAAAACTTTATAGTAACAGAAAC-3′. A total of 50 ng of pmiR-RB-ReportTM-Stra8 3′UTR and pBoule-IRES2-GFP or pIRES2-EGFP (negative control) was co-transfected into HeLa cells in a 48-well plate using Lipofectamine2000 (Invitrogen). After 48 h, all the target validation assays were performed with the dual-luciferase reporter system (Vigorous Biotechnology, Beijing), according to the manufacturer's instructions.

WESTERN BLOTTING

Total cell extracts were prepared from mGSCs in the transfected Boule or not, and proteins were extracted in 1× SDS–PAGE sample loading buffer. Total cell proteins were resolved by SDS–PAGE, transferred to PVDF membrane, and probed with β-actin (1:1,000, Beyotime, Haimen, Jiangsu, China), Stra8 (1:1,000, Abcam), Horseradish peroxidase-conjugated anti-rabbit was used as a secondary antibody (1:1,000, Beyotime). The detection was performed using the Thermo Scientific Pierce ECL Western blotting substrate (Thermo Scientific) [Cao et al., 2011]. The results were analyzed by Tanon-410 automatically gel imaging system (Shanghai Tianneng Corporation, China).

5-BROMO-2-DEOXYURIDINE (BRDU) INCORPORATION ASSAY

The proliferation of mGSCs was assayed by Brdu incorporation, performed similarly to previous reports [Dyce et al., 2004], but with some modifications. First, cells were fixed in 4% PFA for 15 min at room temperature and washed three times, for 10 min each with PBS (pH 7.4) containing 0.1% Triton X-100. The cells were then washed for three times in PBS (pH 7.4) alone. Anti-Brdu (1:100, Santa Cruz) dissolved in 0.1 M PBS (pH 7.4) containing 5% normal goat serum was added and the cells were incubated overnight at 4°C. Cells were washed in PBS (pH 7.4) for three times, and then incubated with corresponding secondary antibody (1:500, beyond time) for 1 h at room temperature. Three more washes were carried out and cells

were visualized under a Leica fluorescent microscope and analyzed for Brdu uptake. Manual counting under fluorescent microscope made the rate of Brdu positive cells.

STATISTICAL ANALYSIS

The data are presented as mean ± SEM. Differences in the expression of specific markers were evaluated using Student's *t*-test (Excel, Microsoft Corporation, Redmond, WA). Results of the different treatments were considered significantly different when a P value < 0.05 was obtained.

RESULTS

EXPRESSION PATTERN OF BOULE IN DAIRY GOAT TESTIS

Semi-quantitative and quantitative RT-PCR analysis had shown that Boule was specifically expressed in testis, but not expressed in kidney, lung, muscle, liver, heart, and fat. Boule was specifically expressed in adult dairy goat testis and had an extremely low-expression level in fetal period and puberty testis. In adult testicular tissue, the expression level of Boule in normal adult testis was significant higher than that in azoospermia and male intersexes testis analyzed by QRT-PCR (Fig. 1A,B).

The results of immunofluorescence manifested that expression level of Boule was significantly enhanced in adult dairy goat testis. Conversely, the expression of Boule was week or not expressed in fetal period, puberty and adult azoospermia testis. The percentage of Boule positive spermatogonia cells in adult testis was significant higher than azoospermia and fetal period, puberty testis. The Boule was located in the cytoplasm of spermatogonia cells (Fig. 1C).

IDENTIFICATION OF DAIRY GOAT BOULE GENE

To further investigate the function of Boule in dairy goat testis, we cloned the 1,091 bp CDs of Boule from adult Guanzhong dairy goat testicular tissue by RT-PCR (Fig. 2A). The cloned Boule was identified by sequencing (Fig. 2B), and has shown that the cloned Boule was identical with the CDS of Boule in NCBI GenBank (NM_001102115). Furthermore, we aligned the cloned sequence with Boule CDS of *B. taurus*, *H. sapiens*, and other species. The results show that the cloned Boule of adult Guanzhong dairy goat was highly homologue with that of *Caprine*, *B. taurus*, *M. mulatta*, and *H. sapiens*. The dairy goat Boule shares 99% homologue with that of *Caprine–B. taurus*, and 98% with that of *M. mulatta–H. sapiens* (Fig. 2C,D).

A homology comparison of Boule CDS showed that the Boule CDS of Guanzhong dairy goat shared high similarity with other species and phylogenetic tree was depicted (Fig. 2C,D).

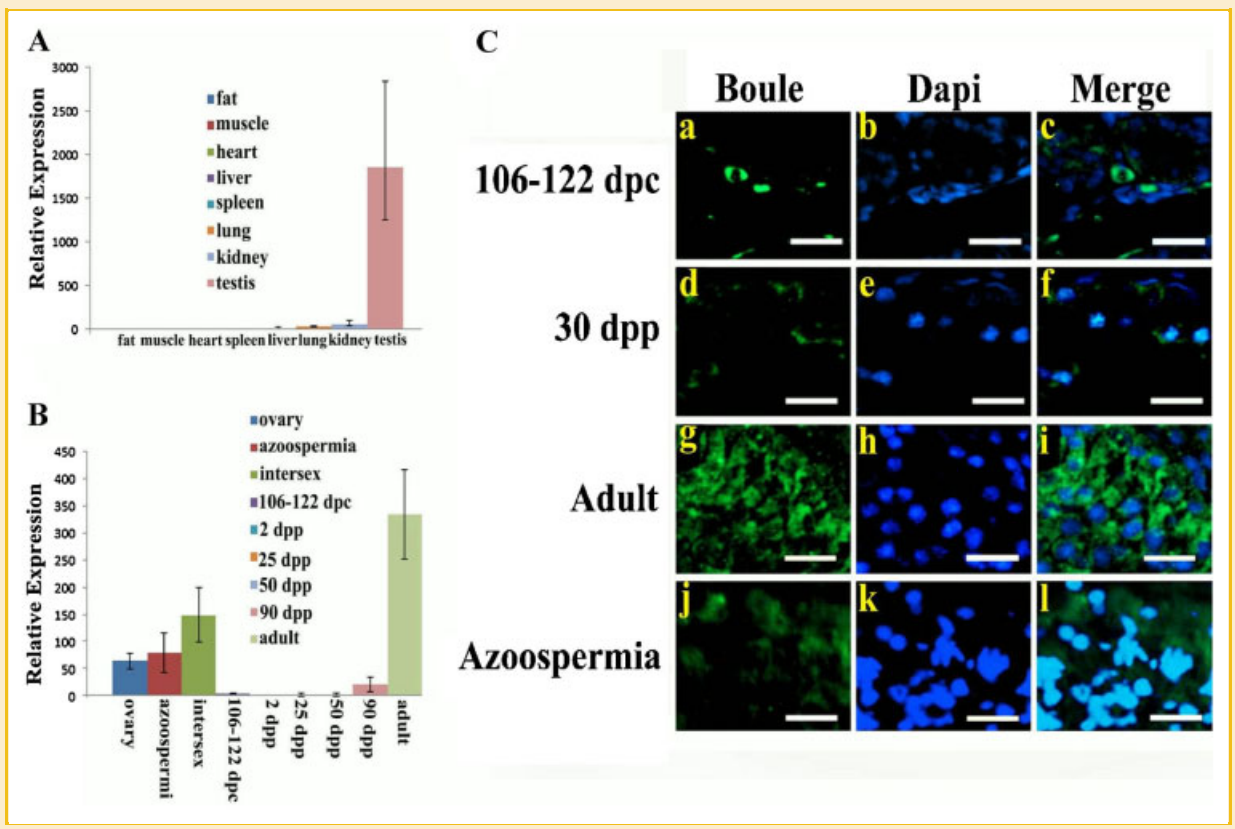

Fig. 1. Expression pattern of Boule in Guanzhong dairy goat testis. A: QRT-PCR analysis has shown that Boule was specifically expressed in testis. B: Boule was specifically expressed in adult dairy goat testis, and the level of Boule in normal testis is significantly higher than younger and immature goats, and azoospermia and male intersex testis analyzed by QRT-PCR. C: Boule was significantly increased in adult dairy goat testis analyzed by immunofluorescence. The percentage of Boule positive spermatogonia cells in adult testis was significant higher than that of azoospermia and fetal period (106–122 dpc), 30 dpp goat testis.

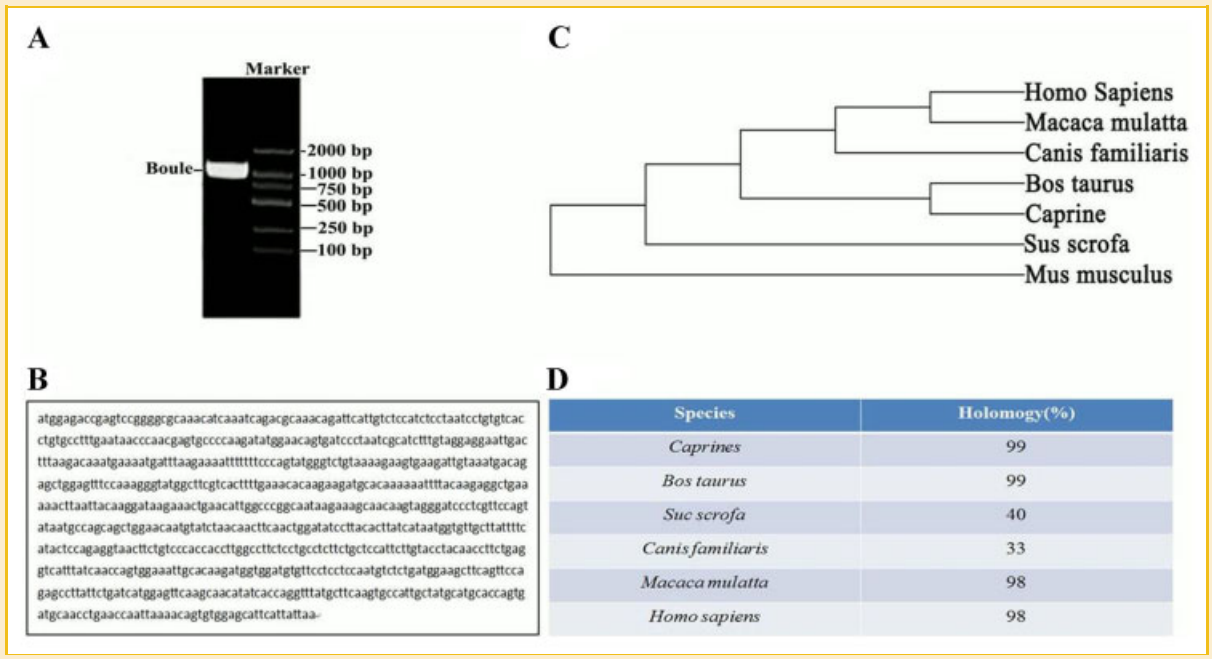

Fig. 2. Identification of Guanzhong dairy goat Boule gene. A: The Boule was cloned. B: The sequence of Guanzhong dairy goat Boule. C: The phylogenetic trees of Boule. D: A homology comparison of Boule CDS show that Boule was conserved.

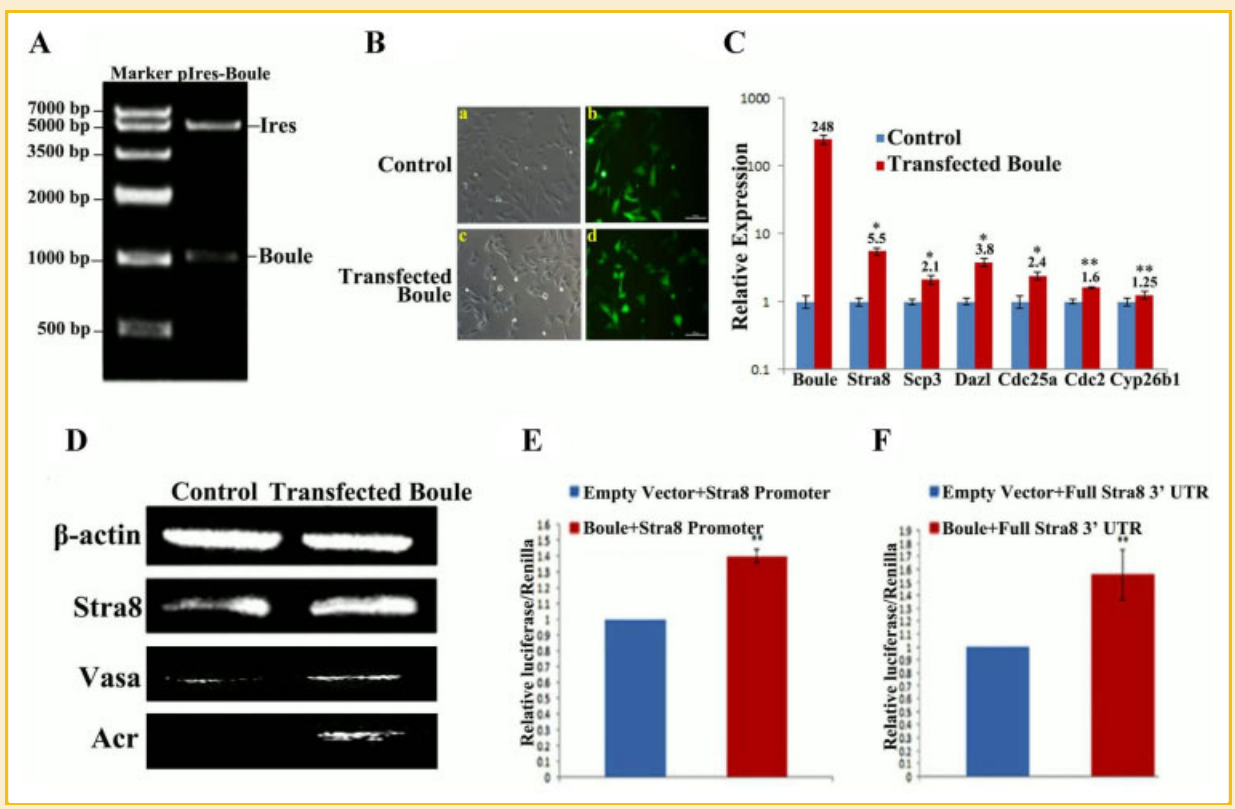

Fig. 3. Over-expression of Boule in dairy goat mGSCs. A: The pBoule-IRES2-GFP was identified by restricted enzyme digestion. B: GFP positive GSCs were observed with fluorescent microscope. (a&b): mGSCs were transfected with pIRES2-GFP (a, Phase contrast; b, Fluorescence microscope); (c&d): mGSCs were transfected with pBoule-IRES2-GFP (c, Phase contrast; d, Fluorescence microscope). C: The expression level of Boule in pBoule-IRES2-GFP transfected group was significantly increased compared with the pIRES2-GFP group analyzed by QRT-PCR. Additionally, the expression of meiosis genes: Stra8, Scp3, Cdc25a, Cdc2 and germ cell marker–Dazl, and Cypb26b1 were remarkably up-regulated in pBoule-IRES2-GFP transfected group. D: The protein level of Vasa, Star8 and Acr were also significantly increased by over-expression of Boule. E: The level of Stra8 was increased 50% in pBoule-IRES2-GFP group compared with control analyzed by pStra8 promoter–LUC Assay. F: The expression of Stra8 in cells transfected with pBoule-IRES2-GFP was increased 50% compared with control analyzed by dual-Luciferase® Reporter Assay; $^{*}P < 0.05$, and $^{**}P < 0.01$.

TRANSFECTION pBOULE-IRES2-GFP RECOMBINATION PLASMID INTO DAIRY GOAT mGSCs

The Boule CDS was obtained and the pBoule-IRES2-GFP plasmid was verified by *Sal*I, *Eco*RI double digestion (Fig. 3A). The cultured dairy goat mGSCs maintained typical GSC and ES-like colonies [Hua et al., 2011; Zhu et al., 2012], and the cells expressed the characteristic markers analyzed by RT-PCR and immunofluorescence (Supplementary Fig. 1). Then, pBoule-IRES2-GFP and pIRES2-EGFP were transfected into dairy goat mGSCs. The results demonstrated that pBoule-IRES2-GFP was successfully transfected into dairy goat mGSCs (Fig. 3B). The expression level of Boule in pBoule-IRES2-GFP transfected group was significantly higher than that in the pIRES2-EGFP group analyzed by QRT-PCR (Fig. 3C). Simultaneously, the expression level of meiosis genes: Stra8, Scp3, Cdc25a, Cdc2 and germ cell marker—Vasa was remarkably up-regulated in over-expression of Boule compared with control (Fig. 3C). The protein of Vasa, Star8 and Acr were also increased by over-expression of Boule (Fig. 3D). Furthermore, the percentage of mGSCs positive for Vasa, Stra8 and Acr was significantly increased by Boule, however, Nanos2 was inhibited by Boule analyzed by immunofluoresce staining (Supplementary Fig. 2).

BOULE REGULATE STRA8 AT BOTH TRANSCRIPTIONAL LEVEL AND POST-TRANSCRIPTIONAL LEVEL

To determine whether Boule directly regulate Stra8 at transcriptional level, we analyzed the expression of Stra8 through pStra8-Luc 3′UTR construct transfection. The results have shown that the level of Stra8 was increased 50% in pBoule-IRES2-GFP group compared with control analyzed by pStra8 promoter-LUC Assay (Fig. 3E).

To test whether Boule regulates Stra8 at post-transcriptional level, pmiR-RB-ReportTM-Stra8 3′UTR and pBoule-IRES2-GFP or pIRES2-GFP (negative control) were co-transfected into HeLa cells. The results showed that the expression of Stra8 in cells transfected with pBoule-IRES2-GFP was increased 50% compared with Control analyzed by dual-Luciferase® reporter assay (Fig. 3F). These results suggest that Boule up-regulate the translation of Stra8 through combination 3′UTR of its mRNA (Fig. 3F). Western blotting has shown that the protein level of Stra8 was also significantly increased in Boule over-expressed cells 48 h after transfection compared with control (Fig. 3D). Additionally, over-expression of Boule has not changed the proliferation of dairy goat mGSCs analyzed by Brdu incorporation assay (Supplementary Fig. 3).

TRANSFECTION PCDC25A-IRES2-GFP INTO DAIRY GOAT mGSCs

Our results demonstrated that Boule directly regulate Stra8, and to control the meiosis (Fig. 3E). Previous studies had shown that Boule initiates MPF then stimulate meiosis by regulating Cdc25a and depholoyation Cdc2 [Xu et al., 2003]. To investigate, whether Boule activate Cdc25a and regulate Stra8, we cloned dairy goat Cdc25a and constructed pCdc25a-IRES2-GFP (Fig. 4A,B). The Cdc25a transfection assay showed that the level of Stra8 in mRNA and protein was not clearly changed by Cdc25a analyzed by QRT-PCR and Western blotting (Fig. 4C,D). These results suggest that Boule might directly regulate Stra8, but not through Cdc25a.

EXPRESSION OF SPECIFIC MARKERS IN MGSCS INDUCED BY RA, OVER-EXPRESSED BOULE AND STRA8

The expression of Boule and Stra8 was significantly promoted by 10^{-5} and 10^{-6} M retinoic acid (RA) (Fig. 5A). Meanwhile, the

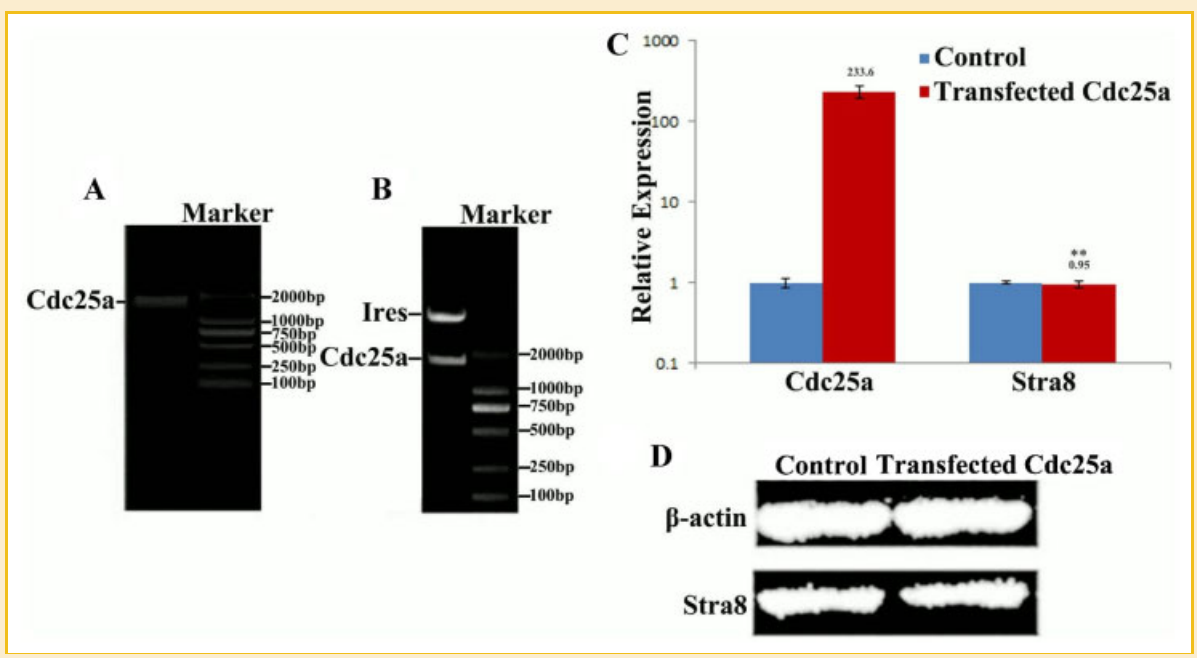

Fig. 4. Transfection pCdc25a-IRES2-GFP recombination plasmid into dairy goat mGSCs. A: Cdc25a was cloned. B: The recombination plasmid pCdc25a-IRES2-GFP was identified by restricted enzyme digestion. C: QRT-PCR analysis the effect of over-expression of Cdc25a. D: Western blotting analysis the effect of over-expression of Cdc25a; $^{**}P < 0.01$.

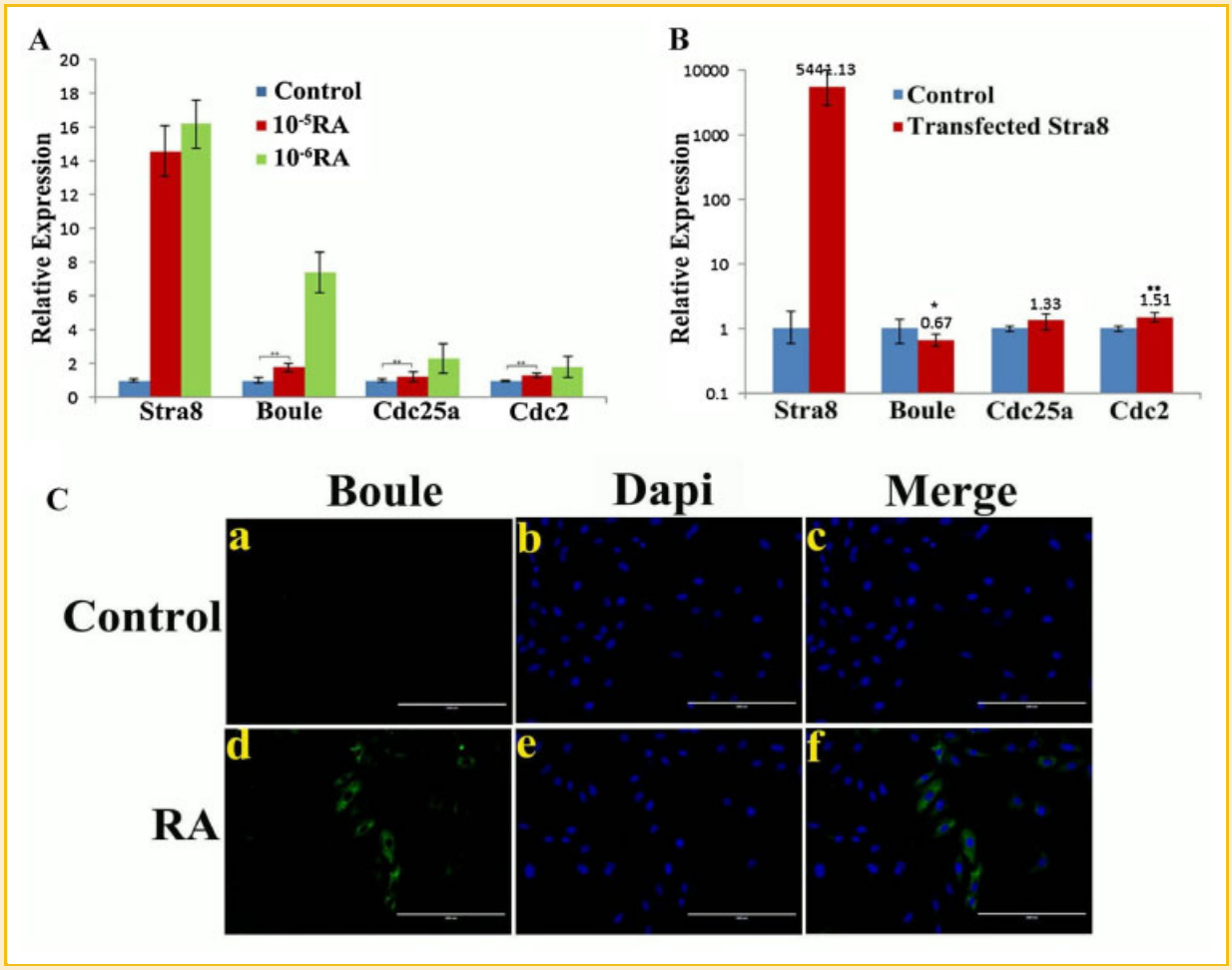

Fig. 5. Expression of specific genes in induced by RA and over-expression of Stra8. A: QRT-PCR analysis the effect of RA induction. B: QRT-PCR analysis the effect of Stra8 over-expression. C: Boule protein was activated by RA induction analyzed by immunofluorescence; $^{*}P < 0.05$, and $^{**}P < 0.01$.

Cdc25a and Cdc2 were mildly promoted by RA. The effects of 10^{-6} RA on the up-regulation of Boule and Stra8 was stronger than 10^{-5} M RA (Fig. 5A). The Boule protein was also activated by 10^{-6} RA analyzed by immunoflorescence (Fig. 5C) however, the expression of Boule was not affected by over-expression of Stra8 and Cdc25a. Cdc2 were little increased by over-expression of Stra8 (Fig. 5B).

DISCUSSION

Boule is a highly conserved regulator of meiosis in Drosophilae, mammalian infertile and display meiotic arrest in the male-germ cells [Escalier, 2001; Luetjens et al., 2004; Li et al., 2011]. Boule is specifically expressed in mammalian testicular tissue and is a critical regulator during the meiosis progress of spermatogenesis. In spermatogenetic malfunction patients, Boule expression was significantly reduced. We first identified that the expression of Boule in adult dairy goat testicular tissue was higher than that of other ages, which was in consistent with previous studies in mice and human, and confirmed its specific expression in the male testis [Li et al., 2007, 2011]. The results indicate that Boule was closely related to dairy goat spermatogenesis and meiosis, specifically expressed in testis, and the expression level in normal testis was significantly higher than that in azoospermia and male intersex testis. These results first demonstrated that the function of Boule in goat was similar as that in Drosophila, mice, Human, and other species [Gromoll et al., 1999; Li et al., 2007, 2011; Shah et al., 2010].

Boule, a member of the human Daz (deleted in azoospermia) gene family, within primates, mammals and metazoans [Gromoll et al., 1999; Shah et al., 2010]. Boule protein level is critical for spermatogenesis, which was beneficial for the treatment of male infertility [Zhang et al., 2009]. In our study, we first cloned the dairy goat Boule CDS and found the sequenced Guanzhong dairy goat Boule CDS shared a high homologue with *Caprine*, *B. taurus*, *M. mulatta*, and *H. sapiens*. The sequence divergence of Boule is unexpectedly low and that rapid evolution is not detectable [Xu et al., 2003; Kostova et al., 2007; Li et al., 2007; Zhang et al., 2009; Shah et al., 2010].

Boule possesses a RNA binding domain, a RNA recognition domain and Daz repeats [Tsui et al., 2000; Lee et al., 2006]. Studies have showed that over-expression human Boule can advance

meiosis in infertile Boule mutant flies [Xu et al., 2003]. Human Dazl functions in PGC formation, whereas its' closely related genes Daz and Boule promote later stages of meiosis and development of haploid gametes [Kee et al., 2009]. In this study, we first demonstrated that Boule directly up-regulated the expression of Stra8. Stra8 is essential for meiotic entry in both male and female germ cells [Kimble, 2011], which was the specific target gene of RA. Over-expression of Boule significantly enhanced the expression of meiosis related Stra8, Scp3 with different degrees, which illustrate Boule mRNA status affects the meiosis process of male-germ cells. The expression level of Stra8 protein was elevated in Boule transfected cells analyzed by Western blotting. Luciferase reporter assay and Dual-Luciferase® Reporter Assay demonstrated that the level of Stra8 was increased 50% in pBoule-IRES2-GFP group compared with control. These results suggested that Boule up-regulate the translation of Stra8 through combination 3′UTR of Stra8. The Boule and Stra8 are specifically up-regulated by RA. These results further provide a new mechanism on Boule promotion the initiation of meiosis.

The expression alteration impacted the germ cells undergoing the first meiosis and affected the primary spermatocyte stage of spermatogenesis [Truong et al., 2003]. The expression of Boule is closely related with spermatocytes meiosis as a key regulator in meiosis [Haag, 2001; Xu et al., 2003; Joiner and Wu, 2004]. We also transfected the Boule gene into mice-spermatogonia cell line-GC1 and mouse male-germline stem cell (mGSCs) and obtained the similar results as in goat mGSCs but a less significant up-regulation of meiosis related genes (data not shown).

How does Boule regulate meiosis? Studies have showed in Drosophilae that Boule was expressed at the prophase of the first meiosis and regulate Twine (phosphates of Cdc25) expression and then initiate MPF, such as Cdc2/cyclin B complex, which is the key factor of G2-M transition and the first meiosis [Xu et al., 2003]. Thus, in Drosophilae, Boule regulates Twine to control meiosis [Maines and Wasserman, 1999]. The pathways regulate germ cells appear to be conserved broadly [Xu et al., 2003]. Our results showed that Boule regulate the meiosis through the up-regulation of Stra8 in mRNA and protein, however, the over-expression of Cdc25 do not affect Stra8. These results demonstrated that Boule regulate the meiosis through a new pathway [VanGompel and Xu, 2010, 2011]. Here, we suppose a hypothesis on the regulation pathway of mammalian meiosis by Boule (Fig. 6).

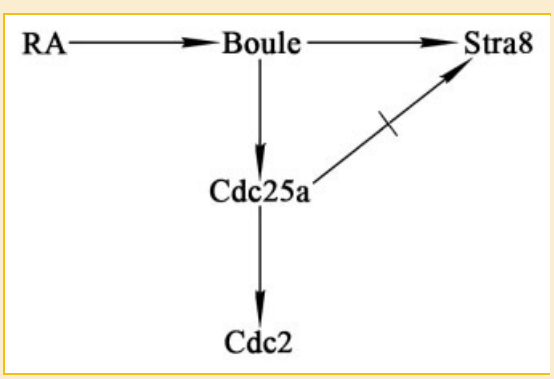

Fig. 6. Summary of the role of Boule in mammalian meiosis. Stra8 was regulated by Boule. Boule and Stra8 was activated by RA, Boule regulated the Cdc25a, then activated Cdc2. However, Cdc25a did not affect Stra8.

In this study, we first clarified the expression pattern of Boule in dairy goat testis and found Boule is required for the meiosis and spermatogenesis in dairy goat testis. Over-expression of Boule in dairy goat mGSCs resulted in the elevation of meiosis related genes: Stra8, Scp3, Cdc25a, Cdc2 and Vasa. This provided a model for further investigation the mechanisms of Boule in spermatogenesis and an approach to treat azoospermia or male infertility.

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