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—Original Article—

NLRP9B protein is dispensable for oocyte maturation and early embryonic development in the mouse

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Abstract. *Nlrp9a*, *Nlrp9b* and *Nlrp9c* are preferentially expressed in oocytes and early embryos in the mouse. Simultaneous genetic ablation of *Nlrp9a* and *Nlrp9c* does not affect early embryonic development, but the function of *Nlrp9b* in the process of oocyte maturation and embryonic development has not been elucidated. Here we show that both *Nlrp9b* mRNA and its protein are expressed in ovaries and the small intestine. Moreover, the NLRP9B protein was restricted to oocytes in the ovary and declined with oocyte aging. After ovulation and fertilization, NLRP9B protein was found in preimplantation embryos. Confocal microscopy demonstrated that it was mainly localized in the cytoplasm in the oocytes and blastomeres. Thus, this protein might play a role in oocyte maturation and early embryonic development. However, knockdown of *Nlrp9b* expression in GV-stage oocytes using RNA interference did not affect oocyte maturation or subsequent parthenogenetic development after *Nlrp9b*-deficient oocytes were activated. Furthermore, *Nlrp9b* knockdown zygotes could reach the blastocyst stage after being cultured for 3.5 days *in vitro*. These results provide the first evidence that the NLRP9B protein is dispensable for oocyte maturation and early embryonic development in the mouse.

Key words: Early development, Mouse, NLRP9B protein, Oocyte maturation

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There are 14 members of the *NLRP* gene family in primates, while 20 members have been identified in the mouse, with *Nlrp1*, *Nlrp4* and *Nlrp9* showing lineage-specific replication. However, *Nlrp7*, *Nlrp8*, *Nlrp11* and *Nlrp13* might have been lost during mouse evolution and have not yet been identified.

Phylogenetic analysis has shown that the *Nlrp* gene family has divided into two clades involving immunization- and reproduction-related functions in mice [1]. Initially, research on this gene family focused on apoptosis and inflammatory signaling pathways. Nevertheless, further studies have shown that some *Nlrp* genes also play a significant role in reproduction [2–6]. The reproduction-related genes are mainly expressed in the ovary and play vital roles in oogenesis and preimplantation embryo development [7–15]. Thus, investigation of the functions of reproduction-related genes is of great significance in understanding the mechanism of oogenesis, oocyte maturation and early embryonic development in the mouse.

In silico methods revealed that *Nlrp2*, *Nlrp4a-Nlrp4g*, *Nlrp5*/*Mater*, *Nlrp9a–Nlrp9c* and *Nlrp14* are reproduction-related genes in the mouse [1]. *Nlrp5*/*Mater* was one of the first genes to be identified as having a maternal effect, as an *Nlrp5* knockout experiment showed that *Nlrp5*-deficient embryos arrested at the 2-cell stage, causing female infertility, even though the formation of follicles, oocyte maturation and fertilization were all normal [2]. Further research showed that the NLRP5/MATER, FLOPED, TLE6 and FILIA proteins form a subcortical complex in the oocyte that plays an important role in the first zygotic cleavage [3, 4]. Knockdown of *Nlrp2* or *Nlrp14* expressions in the zygote led to most zygotes being arrested at the 1-cell to 8-cell stage [6, 9]. Besides *Nlrp2*, *Nlrp5* and *Nlrp14*, *Nlrp4a–Nlrp4g* also show specificity or are mainly expressed in the ovary [14, 16]. The latest research has indicated that the *Nlrp9a–Nlrp9c* transcripts exist predominantly in oocytes and early embryos [15]. Furthermore, simultaneous genetic ablation of *Nlrp9a* and *Nlrp9c* does not affect early embryonic development in the mouse [17]. However, the function of *Nlrp9b* in the process of oocyte maturation and embryonic development in the mouse has not been elucidated.

In this study, RNA interference was employed to investigate the function of *Nlrp9b* in oocyte maturation and early embryonic development in the mouse. The results indicated that *Nlrp9b*-deficient oocytes can undergo normal maturation and that *Nlrp9b* knockdown zygotes could reach the blastocyst stage. These data provide the first evidence that *Nlrp9b* is dispensable for oocyte maturation and early embryonic development in the mouse.

Materials and Methods

Ethics statement

The experimental procedure was approved by the Animal Care Commission of the College of Animal Science, Fujian Agriculture and Forestry University. Adult male and female ICR strain mice were purchased from the Experimental Animal Center of Fujian Medical University (Fuzhou, P. R. China). They were provided with water and mouse chow *ad libitum* and maintained on a 14/10 h light/dark cycle in the Laboratory Animal Facility of the College of Animal

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Chemicals

All chemicals and reagents were obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated. Sterile plastic ware was purchased from Nunclon (Roskilde, Denmark).

Collection of oocytes and preimplantation embryos

In the experiments using germinal vesicle (GV)-stage oocytes, female ICR mice were injected with 10 IU pregnant mare serum gonadotropin (PMSG) by intraperitoneal injection 48 h before collection of oocytes. The ovaries were removed and placed in Hepes-buffered potassium simplex optimized medium (H-KSOM) containing 0.1 mM 3-isobutyl-1-methyl-xanthine to produce meiotic arrest, and oocytes were released by puncturing the edges of the ovaries with hypodermic needles. Metaphase II oocytes were obtained from the oviducts of female mice that had been given a second injection of 10 IU human chorionic gonadotropin 48 h after the PMSG injection. Cumulus masses were released into H-KSOM containing hyaluronidase (1 mg/ml). Parthenogenetic activation of oocytes and collection of preimplantation embryos were performed as described previously [6].

Reverse transcription polymerase chain reaction (RT–PCR) and quantitative real-time RT–PCR analysis

Total RNA was collected from 4-week-old mouse tissues (ovary, small intestine, testis, lung, heart, liver, brain, stomach, spleen and muscle) using RNeasy Mini Kits (Qiagen, Valencia, CA, USA). Complementary (c) DNA was synthesized using a PrimeScript II 1st strand cDNA Synthesis Kit (TaKaRa, Otsu, Japan). Oocytes (10 per group) were lysed, and first-strand cDNA was synthesized directly using a SuperScript® III CellsDirect cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The primer sequences used for RT–PCR and quantitative real-time RT–PCR were 5′–GACTTCACCAGTGACTGTTGTG–3′ (forward) and 5′–CCATACCAGACGAACACCC–3′ (reverse) for *Nlrp9a*, 5′– CACCAGTGACTGTTGTAAGGAC–3′ (forward) and 5′–TTCCTGCTGTTCCATACCAG–3′ (reverse) for *Nlrp9b*, 5′–TTCCAGTGAATCAACATCAGCT–3′ (forward) and 5′–GAACAGGGTAGCAAAGTACCA–3′ (reverse) for *Nlrp9c* and 5′– GCTTGCTGGTGAAAAGGACCTCTCGAAG–3′ (forward) and 5′– CCCTGAAGTACTCATTATAGTCAAGGGCAT–3′ (reverse) for *Hprt1*. The specificity of each primer pair and RT–PCR products were evaluated by electrophoresis and annealing temperature analysis to confirm the absence of primer dimers and to estimate fragment sizes. Quantitative real-time RT–PCR reactions were performed in triplicate for each individual sample in a final volume of 10 µl containing 2 × SYBR Premix ExTaq™ II, 100 nM each primer and 1 µl of cDNA on an ABI PRISM 7700 Sequence Detection System (Life Technologies, Carlsbad, CA, USA). The cycling conditions comprised 1 min preincubation at 95 C and 40 cycles at 95 C for 5 sec and 56–60 C for 30 sec. Expression of the target gene in each run was normalized to the housekeeping gene *Hprt1*. Relative expression was estimated using the $2^{-\Delta\Delta CT}$ method [18].

Western blotting

Protein samples from mouse tissues, oocytes, and preimplantation embryos were homogenized and solubilized in RIPA lysis buffer (P0013B, Beyotime Institute of Biotechnology, Jiangsu, P. R. China). The tissue lysates were centrifuged at 12,000 *g* for 20 min at 4 C. The supernatant fractions were collected, and the protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein obtained from tissues or oocytes and preimplantation embryos were separated in 10% polyacrylamide gels containing 0.1% SDS and transferred to hydrophobic PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with Tris-buffered saline (TBS pH 7.4)/0.1% Tween 20 (TBST) containing 5% dry milk for 4 h at room temperature, incubated with antibodies against NLRP9B (NBP2-24661, Novus Biologicals, Littleton, CO, USA; 1:100) overnight at 4 C and then washed three times in TBST. A horseradish peroxidase-conjugated secondary antibody was added 2 h prior to processing using an ECL (enhanced chemiluminescence) Advance Western Blotting detection system (Pierce). β-Actin was used as a loading control.

Immunohistochemistry

Mouse ovaries were fixed in 4% paraformaldehyde overnight, embedded in paraffin wax and sectioned at 6 µm. These sections were then deparaffinized, rehydrated and heated in 10 mM sodium citrate buffer (pH 6.0) with microwaves for antigen retrieval. Immunohistochemistry of ovarian tissues using UltraSensitive™ SAP IHC kits (Fuzhou Maixin Biotech, P. R. China) was performed as described previously [6]. In brief, sections were incubated with 10% nonimmune goat serum and then with an anti-NLRP9B polyclonal antibody (1:100) overnight at 4 C, followed by reaction with a biotinylated goat anti-rabbit secondary antibody. After several washes, streptavidin–peroxidase and a diaminobenzidine substrate solution were applied to the sections. Negative controls were performed via processing sections as above with the absence of the primary antibody.

Immunofluorescence

Oocytes and embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature, washed and permeabilized with 0.2% Triton X-100 in PBS for 20 min. Samples were first incubated in PBS containing 3% BSA for 1 h to block nonspecific interactions. Immunofluorescence staining was performed by incubating the samples with an antibody against NLRP9B (1:200) overnight at 4 C, followed by an incubation in Alexa Fluor 488-conjugated goat anti-rabbit (Beyotime, A0428) diluted 1:500 for 2 h. DNA was stained with DAPI (C1005, Beyotime Institute of Biotechnology) for 5 min. Fluorescence was detected using a Zeiss LSM 510 confocal microscope equipped with differential interference contrast optics (Carl Zeiss, Thornwood, NY, USA).

Electroporation of customized short interfering (si)RNA

A mixture of three target-specific 19–25 nt siRNA that specifically target the *Nlrp9b* transcripts was purchased from Santa Cruz Biotechnology (sc-149819, Dallas, TX, USA). Electroporation was employed to deliver siRNA into GV-stage oocytes and zygotes as described previously [6]. Briefly, GV-stage oocytes and zygotes

were incubated in prewarmed acid Tyrode's solution for 12 sec to weaken the zona pellucida. The siPORT *Amine* Transfection Agent (AM4502, Ambion, Austin, TX, USA) and the negative control siRNA (Silencer® negative control No. 1, AM4611, Ambion) or the custom *Nlrp9b* siRNA were diluted in Opti-MEM® I (Invitrogen) medium according to the manufacturers' protocols, respectively. After 10 min, the diluted siRNA and the siPORT *Amine* Transfection Agent were combined and incubated for 10 min at room temperature to form transfection complexes. The transfection complexes and oocytes or zygotes were added to a flat electrode chamber and arranged linearly between two electrodes. The electroporation parameters were DC 20 volts/1 msec pulse length, 3 pulses and 0 repeats. The electroporation control (EP control) was performed as mentioned above in the absence of siRNA. A negative control siRNA was used as the negative control.

After electroporation, GV-stage oocytes were cultured in MEM supplemented with 3 mg/ml bovine serum albumin (MEM-BSA) and 0.1 mM 3-isobutyl-1-methyl-xanthine for 24 h, washed three times and incubated in MEM-BSA for another 12 h, followed by calculation of the maturation rates of the oocytes. Zygotes were incubated in KSOMaa medium containing 4 mg/ml bovine serum albumin (KSOMaa-BSA) in a humidified atmosphere of 5% CO_2/95% air at 37 C.

Statistical analysis

All experiments were repeated at least three times. The results are presented as the mean ± SEM. Data were analyzed by one-way ANOVA and LSD tests using the SPSS version 13.0 software (SPSS, Chicago, IL, USA). Differences were considered significant when $P < 0.05$.

Results

Tissue distribution of Nlrp9b

RT–PCR analysis detected transcripts of *Nlrp9b* in ovaries and the small intestine (Fig. 1A). The expression pattern of NLRP9B protein, as determined by western blotting, was similar to that determined by RT–PCR assay (Fig. 1B).

NLRP9B expression was restricted to oocytes and declined with oocyte aging in the ovary

NLRP9B protein was detected in oocytes by immunohistochemistry (Fig. 2A). Furthermore, its expression declined with oocyte aging (Fig. 2B).

Expression and localization of NLRP9B in preimplantation embryos

Western blotting confirmed that the NLRP9B protein was detected in all preimplantation embryos, although its expression declined with further development (Fig. 3A). Confocal microscopy demonstrated a mainly cytoplasmic localization of the NLRP9B protein in mature oocytes and preimplantation embryos (Fig. 3B).

Effect of Nlrp9b knockdown on oocyte maturation

As shown in Fig. 4A, the expression of *Nlrp9b* was downregulated in a dose-dependent manner, and a concentration of 600 nM siRNA

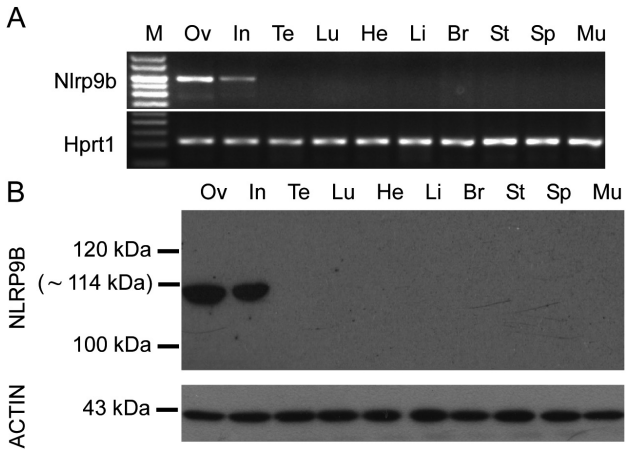

Fig. 1. Expression of *Nlrp9b* transcripts and proteins in different mouse tissues. (A) Reverse-transcription polymerase chain reaction (RT-PCR) was applied to total RNA extracted from the 4-week-old mouse ovary (Ov), intestines (In), testis (Te), lung (Lu), heart (He), liver (Li), brain (Br), stomach (St), spleen (Sp), and muscle (Mu). *Hprt1* was used as a control. (B) Western blotting analyses with total proteins extracted from the 4-week-old mouse ovary (Ov), intestines (In), testis (Te), lung (Lu), heart (He), liver (Li), brain (Br), stomach (St), spleen (Sp) and muscle (Mu). Molecular masses (kDa) are indicated on the left. ACTIN was used as an internal control.

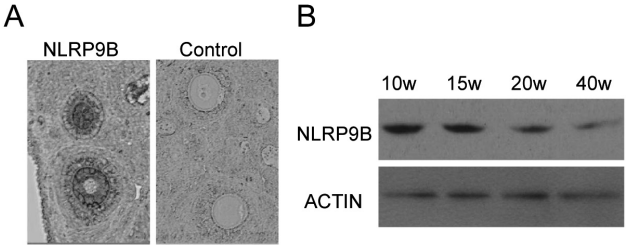

Fig. 2. Expression of NLRP9B proteins in the ovary and GV-stage oocytes. (A) Immunohistochemistry of ovarian sections using the anti-NLRP9B antibody. The original magnification was × 100. (B) Detection of NLRP9B proteins in GV-stage oocytes from mouse ovaries at 10, 15, 20 and 40 weeks of age using western blotting. ACTIN was used as an internal control.

was suitable in terms of efficiency. The *Nlrp9b* knockdown effect was specific, because the expressions of *Nlrp9a* and *Nlrp9c* were not affected by the delivery of *Nlrp9b* siRNA (Fig. 4B). Furthermore, NLRP9B protein levels declined sharply in mature oocytes 24 h after electroporation with *Nlrp9b* siRNA compared with control groups (Fig. 4C). However, the decline of *Nlrp9b* transcripts and protein in oocytes did not affect oocyte maturation (Fig. 4D). Mature oocytes obtained from oocytes electroporated at the GV stage were subjected to parthenogenetic activation and then cultured in KSOMaa-BSA medium. The 2-cell and 4-cell stage parthenogenetic embryos obtained from mature knockdown oocytes were collected and subjected to western blot analysis. We found that NLRP9B protein was not detected in the knockdown group, while the protein was detected in control

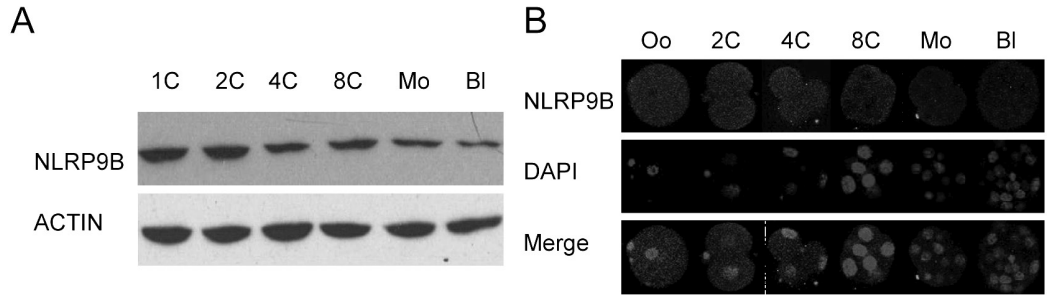

Fig. 3. Expression and localization of NLRP9B proteins in preimplantation embryos. (A) Western blotting of lysates isolated from preimplantation embryos. Oo, mature oocytes; 1C, 1-cell stage; 2C, 2-cell stage; 4C, 4-cell stage; 8C, 8-cell stage; Mo, morula; Bl, blastocyst. ACTIN was used as an internal control. (B) Confocal microscopic images of oocytes and preimplantation embryos. Each sample was counterstained with DAPI to visualize DNA (blue). The original magnification was × 200.

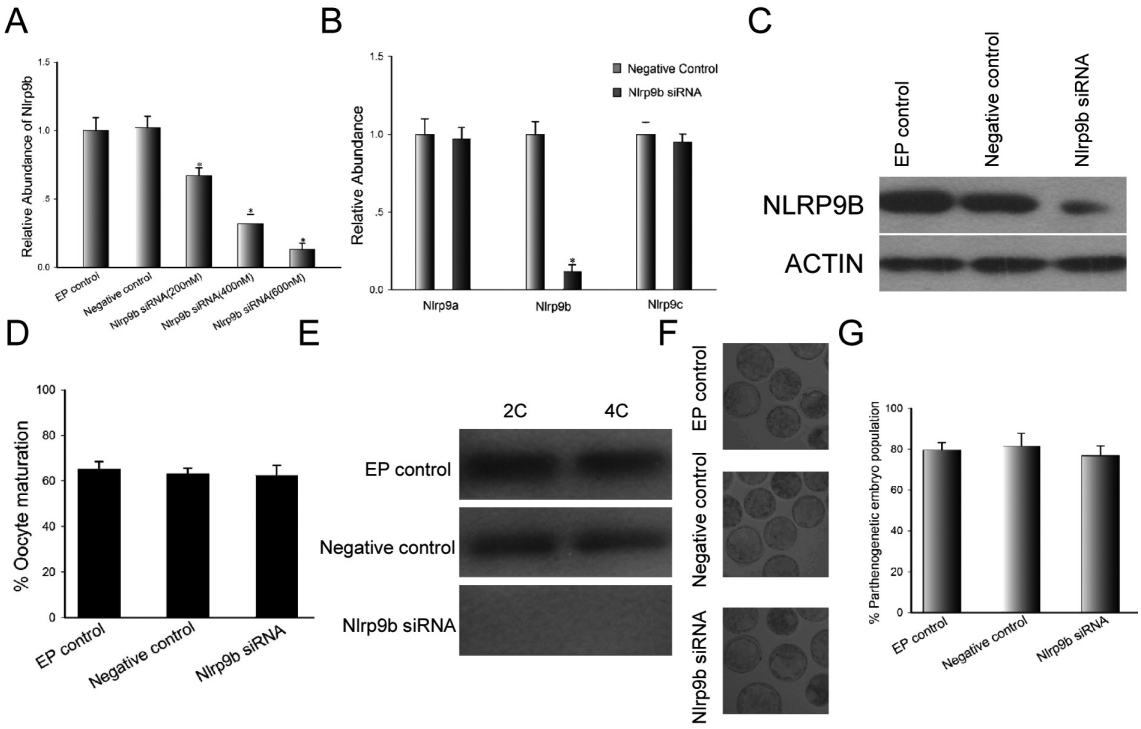

Fig. 4. GV-stage oocyte maturation after electroporation with *Nlrp9b* siRNA. (A) Relative abundance of *Nlrp9b* transcripts following electroporation of GV oocytes with custom siRNA at different concentrations (200 nM, 400 nM and 600 nM) and culture for 48 h. The data were normalized to the electroporation control (EP control). Statistical comparisons were made using ANOVA and LSD tests (* $P < 0.05$). (B) *Nlrp9a*, *Nlrp9b* and *Nlrp9c* gene expression by qRT-PCR in mouse oocytes at 24 h after electroporation with *Nlrp9b* siRNA (600 nM). Results were normalized to the negative control (600 nM). * $P < 0.05$. (C) Western blot analysis of mouse oocytes derived from the EP control, negative control (600 nM) and *Nlrp9b* siRNA group (600 nM) after 24 h culture. ACTIN was used as an internal control. (D) Oocyte maturation rate following electroporation of GV-stage oocytes in the EP control, negative control (600 nM) and *Nlrp9b* siRNA group (600 nM). (E) Western blot analysis of mouse parthenogenetic embryos derived from the EP control, negative control (600 nM) and *Nlrp9b* siRNA group (600 nM). 2C, 2-cell stage; 4C, 4-cell stage. (F) Morphological appearance of parthenogenetic embryos obtained from the control and *Nlrp9b* siRNA groups after culture for 3.5 days. The original magnification was × 100. (G) Percentage of parthenogenetic embryos derived from the control and *Nlrp9b* siRNA groups after culture for 3.5 days.

groups (Fig. 4E). The embryo development stages and morphological appearances of the embryos after they were cultured for 3.5 days are presented in Fig. 4F. Parthenogenetic embryos obtained from the control and knockdown groups reached the blastocyst stage, and there was no significant difference between the three groups with regard to the rate of blastocyst formation (Fig. 4G).

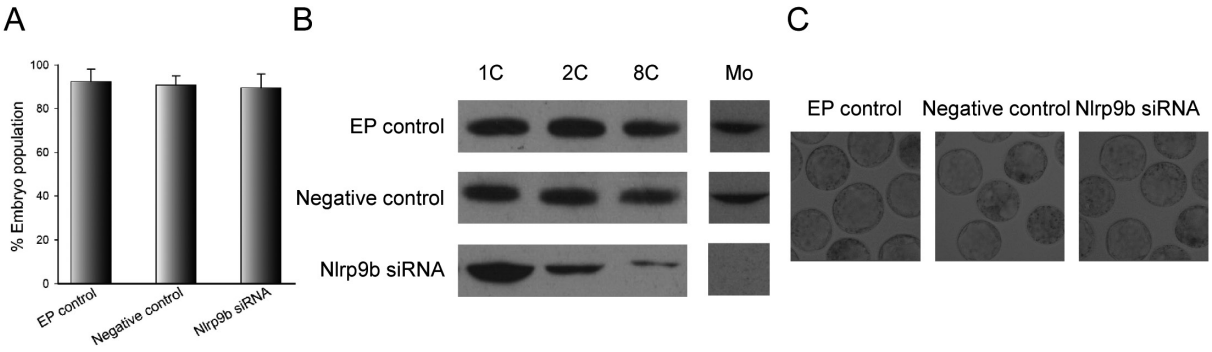

Fig. 5. Development of zygotes after electroporation with *Nlrp9b* siRNA. (A) The blastocyst formation rate of zygotes obtained from the control and *Nlrp9b* siRNA groups after culture for 3.5 days. (B) Western blotting of mouse embryos at 4 h (1 cell), 28 h (2 cells), 52 h (8 cells) and 64 h (morula) after electroporation. (C) Morphology of *Nlrp9b* knockdown embryos after culture for 3.5 days. The original magnification was × 100.

Nlrp9b is dispensable for early embryonic development

To further test the function of *Nlrp9b* in preimplantation development, the zygotes were electroporated with *Nlrp9b* siRNA and cultured in KSOMaa-BSA medium for 3.5 days. As shown in Fig. 5A and 5B, the rates of blastocyst formation showed no obvious differences between the two control groups and the *Nlrp9b* knockdown group, although the introduction of *Nlrp9b* siRNA led to a notable reduction in the NLRP9B protein level. Thus, embryos obtained from all three groups reached the blastocyst stage successfully (Fig. 5C).

Discussion

Molecular evidence has shown that maternal factors are important for oogenesis and the development of preimplantation embryo in mammals [19–26]. Some of the NLRP proteins transcribed and translated by the *Nlrp* gene family are maternal factors such as NLRP9A, NLRP9B and NLRP9C in the mouse. Surprisingly, female mice obtained from a *Nlrp9a* and *Nlrp9c* simultaneous knockout model could give birth to offspring [17]. However, the developmental potential of *Nlrp9b*-deficient GV-stage oocytes and zygotes is uncertain.

Both *Nlrp9b* transcripts and proteins were expressed in ovaries and the small intestine, unlike *Nlrp2*, which is detected only in mouse ovaries [6]. This pattern differs from *NLRP9*, which is expressed in bovine ovaries as well as testes [27]. We found here that the NLRP9B protein was restricted to oocytes and declined with oocyte aging in the ovary, suggesting that this protein might play an essential role in oogenesis. The expression pattern of *Nlrp9b* in mouse ovaries was similar to that reported for *Nlrp4a*, *Nlrp4f*, *Nlrp4g* and *Nlrp14* [9, 13].

After ovulation and fertilization, the NLRP9B protein was present throughout early embryogenesis, while the protein level diminished gradually with embryo development. This expression pattern was similar to those of NLRP2, NLRP4G and NLRP5 during preimplantation embryo development [6, 8, 16]. Confocal microscopy revealed that the NLRP9B protein was predominantly localized in the cytoplasm of blastomeres. This distribution of NLRP9B protein is similar to those of NLRP2 and NLRP4G [6, 16] and differs from that of NLRP5, which is mainly located in the subcortical regions of oocytes and preimplantation embryos [3, 4].

The expression and accumulation of *Nlrp9b* transcripts and proteins in immature oocytes might reflect a physiological role in the process of oocyte maturation. To test this hypothesis, custom siRNAs targeting *Nlrp9b* transcripts were delivered into GV-stage oocytes. However, these oocytes progressed through the metaphase of meiosis I and emitted the first polar body, suggesting that the NLRP9B protein is dispensable for oocyte maturation. The function of *Nlrp9b* is unlike the known maternal-effect genes *Formin-2* and *basonuclin*, which are required for oocyte maturation [20, 21]. Of course, there is a possibility that a tiny amount of NLRP9B protein was present in knockdown oocytes and enabled the GV-stage oocytes to emit the first polar body. The *Nlrp9b*-deficient embryos obtained from *Nlrp9b* knockdown oocytes or zygotes underwent normal development in the terms of the rate of blastocyst formation, implying that this maternal factor is not required for early embryo development in the mouse. The function of *Nlrp9b* differs from those of *Nlrp2*, *Nlrp5* and *Nlrp14*, which are essential for early embryogenesis, suggesting that different *Nlrp* genes have different functions even though they are mainly expressed in oocytes and early embryos. Moreover, *Nlrp9b* might play a key role in intestinal development or intestinal mucosal immunity, as its transcripts and proteins were also detected in the small intestine. Knockout models for this gene or other targeted inhibition experiments should be carried out to uncover the function of *Nlrp9b* in intestinal development or intestinal mucosal immunity.

In summary, the *Nlrp9b* gene was expressed in ovaries and the small intestine in the mouse. In the ovary, the NLRP9B protein was restricted to oocytes and declined with oocyte aging. After ovulation and fertilization, NLRP9B protein was present throughout early embryogenesis and located in the cytoplasm of blastomeres. Furthermore, targeted gene knockdown experiments indicated that the NLRP9B protein was not required for oocyte maturation or preimplantation embryo development in the mouse, implying that *Nlrp9b* might be an immunization-related gene involved in intestinal mucosal immunity in mice.

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