Involvement of *Nlrp9a/b/c* in mouse preimplantation development

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Abstract

Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing proteins (NLRPs) are central components of the inflammasome. Accumulating evidence has shown that a reproductive clade of NLRPs is predominantly expressed in oocyte to cleavage stage embryos and participates in mammalian preimplantation development as a component of a multiprotein complex known as the subcortical maternal complex (SCMC). *Nlrp9s* belong to the reproductive class of NLRPs; *Nlrp9b* is unique in acting as an inflammasome against rotavirus in intestines. Here we generated mice carrying mutations in all three members of the *Nlrp9a/b/c* gene (*Nlrp9* triple mutant (TMut) mice). When crossed with WT males, the *Nlrp9* TMut females were fertile, but deliveries with fewer pups were increased in the mutants. Consistent with this, blastocyst development was retarded and lethality to the preimplantation embryos increased in the *Nlrp9* TMut females *in vivo*. Under *in vitro* culture conditions, the fertilized eggs from the *Nlrp9* TMut females exhibited developmental arrest at the two-cell stage, accompanied by asymmetric cell division. By contrast, double-mutant (DMut) oocytes (any genetic combination) did not exhibit the two-cell block *in vitro*, showing the functional redundancy of *Nlrp9a/b/c*. Finally, *Nlrp9* could bind to components of the SCMC. These results show that *Nlrp9* functions as an immune or reproductive NLRP in a cell-type-dependent manner.

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Introduction

Maternal-to-zygotic transition (MZT), also referred to as oocyte-to-embryo transition (OET), transforms highly differentiated cell types, such as oocytes, into undifferentiated blastomeres of cleavage-stage embryos. Maternal factors and zygotic genome activation (ZGA) play central roles in MZT (Lu *et al.* 2017, Schultz *et al.* 2018). While maternal products such as mRNAs and proteins are degraded rapidly after fertilization, a number of maternal factors are prerequisites for the progression of preimplantation development. Furthermore, ZGA is a major contributor to the creation of transcriptomes of cleavage-stage embryos. Thus, in mammals, preimplantation embryos sequentially acquire totipotency and pluripotency during MZT (Dang-Nguyen & Torres-Padilla 2015, Leung & Zernicka-Goetz 2015).

Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing proteins (NLRPs; sometimes called NALPs) share a conserved tripartite domain containing an N-terminal pyrin

domain, a central nucleotide-binding oligomerization (NACHT) domain, and a C-terminal leucine-rich repeat (LRR) domain, and are classified into two families: the immune and reproductive NLRP families (Tian *et al.* 2009). While immune NLRPs are expressed mainly in the cells responsible for innate immune responses (Pétrilli *et al.* 2007, Rathinam and Fitzgerald 2016), reproductive NLRPs are expressed predominantly in oocytes and preimplantation embryos. The immune NLRP family proteins are central components of a multiprotein complex inflammasome that is responsible for inflammatory responses. Pathogenic microorganisms and sterile stressors trigger the oligomerization of NLRPs and assembly of other inflammasome components, such as caspases, which ultimately leads to maturation and secretion of the pro-inflammatory cytokines interleukin-1 β (IL-1 β) and IL-18, as well as induction of a form of programmed cell death, termed pyroptosis.

Nlrp5, a member of the reproductive NLRP family also known as MATER, is a maternal factor essential for the progression of preimplantation development in mice. Female mice lacking *Nlrp5* are infertile; when

the oocytes of the *Nlrp5*-mutant females are fertilized with the sperm of WT males, the zygotes never develop beyond the two-cell stage, according to a phenomenon known as 'two-cell block' (Tong *et al.* 2000). *Nlrp5* is localized in the subcortical region of oocytes and preimplantation embryos and forms the subcortical maternal complex (SCMC), which is a large protein complex containing Floped (Ooep), Tle6, Filia (Khdc3) and Padi6 (Li *et al.* 2008). Similar to the *Nlrp5*-mutant females, female mice deficient in *Floped*, *Tle6* and *Padi6* are sterile due to developmental arrest of the zygotes at the two-cell stage (Esposito *et al.* 2007, Li *et al.* 2008, Tashiro *et al.* 2010, Yu *et al.* 2014), thus demonstrating the crucial roles of the SCMC in mouse preimplantation development. Meanwhile, mutations and single-nucleotide variants of human *NLRP5* were found in mothers with molar pregnancy who gave birth to offspring with multi-locus imprinting disorders (Docherty *et al.* 2015). Additionally, mutations and variants of human *KHDC3L* are also associated with familial recurrent hydatidiform moles (Parry *et al.* 2011, Reddy *et al.* 2013, Rezaei *et al.* 2016). Thus, the components of the SCMC are pivotal factors in embryonic development in mice and humans.

Nlrp9 is classified as a reproductive NLRP, based on its expression pattern and homology to the reproductive NLRPs (Tong *et al.* 2000). Nonetheless, it was recently demonstrated that mouse *Nlrp9b* and human *NLRP9* are highly expressed in intestinal epithelium and that the *Nlrp9b*-containing inflammasome restricts rotavirus infection in mouse intestines (Zhu *et al.* 2017). In this study, we generated mice carrying mutations in all three *Nlrp9* genes to explore their function as reproductive NLRPs. The preimplantation embryos developed in the mutant female mice *in vivo* exhibited increased lethality and a delay in reaching the blastocyst stage. Furthermore, the fertilized eggs isolated from the mutant female mice displayed two-cell block in *in vitro* culture conditions. Our finding clearly shows that *Nlrp9* functions not only as an immune NLRP but also as a reproductive NLRP, depending on the cell types.

Materials and methods

Animals

ICR mice were purchased from Japan SLC (Shizuoka, Japan). ICR females were injected with pregnant mare serum gonadotropin (PMSG; ASKA Pharmaceutical, Tokyo, Japan) and human chorionic gonadotropin (hCG; ASKA Pharmaceutical) at 46–48 h intervals and were crossed with ICR males to collect fertilized eggs. ICR females were crossed with vasectomized ICR males to prepare pseudopregnant mice. All animal care and experimental procedures were carried out in accordance with the Guidelines of Animal Experiments of Kitasato University and were approved by the Institutional Animal Care and Use Committee of Kitasato University.

Generation of *Nlrp9*-mutant mice

Two guide RNAs (gRNAs), *gRNAa/c* and *gRNAb/c*, which target the *Nlrp9a/c* and *Nlrp9b/c* genes, respectively, were designed using CRISPRdirect (<https://crispr.dbcls.jp/>). The target sequences of *gRNAa/c* and *gRNAb/c* are 5'-CTCAACCATGGCTCAGACAG-3' and 5'-GCAATGTTGGATTGGGCATC-3', respectively, and are unique in the mouse genome except for *Nlrp9a/b/c*. The gRNAs were synthesized using the MEGAscriptTMT7 *in vitro* transcription system (Thermo Fisher Scientific) and dissolved in Opti-MEM (Thermo Fisher Scientific).

The gRNA and Cas9 protein (Medical & Biological Laboratories, Aichi, Japan) were introduced into one-cell embryos as described, with modifications (Hashimoto & Takemoto 2015, Sakai *et al.* 2019, Kosugi *et al.* 2020). Briefly, one-cell embryos were isolated by removing cumulus cells by treatment with 30 µg/mL hyaluronidase (Sigma-Aldrich) in M2 medium and were then washed and cultured in KSOM (ARK Resource, Kumamoto, Japan) at 37°C and 5% CO₂ until electroporation. KSOM was replaced with Opti-MEM immediately before electroporation. Approximately 20 fertilized eggs were suspended in Opti-MEM containing 200 ng/µl gRNA(s) and 200 ng/µl Cas9 protein, placed between two platinum block electrodes connected to a pulse generator (CUY21Vivo-SQ; BEX, Tokyo, Japan) and maintained on ice until electroporation. Electroporation was carried out at room temperature. The condition for electroporation was 30 V (3 ms ON + 97 ms OFF) × 7. The fertilized eggs were washed several times and cultured in KSOM overnight at 37°C and 5% CO₂. On the following day, two-cell embryos were transferred into the fimbriae tubae of the pseudopregnant mice. The pups were obtained by Caesarean dissection.

Genotyping

The following forward and reverse primer pairs were utilized to amplify the target sequences of individual *Nlrp9* genes (Fig. 1A); 5'-TCATCTGGGTATGGCTTGCT-3' and 5'-AAATGGAGGAGCAGTGGATG-3' for exon 2 of *Nlrp9a* (a target of *gRNAa/c*); 5'-TGGCTTGCTGCAGTATTTCA-3' and 5'-ACTGCCCTAGTGGGAAGGA-3' for exon 2 of *Nlrp9c* (a target of *gRNAa/c*); 5'-TGGACATTGGAGACCAACAC-3' and 5'-CGGCTAAGCTTAAGTCTGTGG-3' for exon 3 of *Nlrp9b* (a target of *gRNAb/c*); 5'-TGGACATCTGAGACCAACACTT-3' and 5'-TGTCTCAGAACACTCAGGCAAT-3' for exon 3 of *Nlrp9c* (a target of *gRNAb/c*). The forward primers were used for Sanger sequencing.

Fertility test

Female mice aged over 6 weeks were crossed with WT males for 3 months. The litter size was examined immediately after delivery, following which the pups were sacrificed.

In vitro development

Female mice were injected with PMSG and hCG and crossed with WT males to collect the fertilized eggs as described above.

Figure 1 Generation of the *Nlrp9* mutant mice and expression of mouse *Nlrp9a/b/c*. (A) The structure of the *Nlrp9a/b/c* genes and the positions of the gRNAs. *gRNAa/c* and *gRNAb/c* correspond to exon 2, encoding the pyrin domain and exon 3, encoding the NACHT domain, respectively. The target sequences are enclosed with lines, and the PAM sequences are underlined. Arrows show the cleavage sites of Cas9. (B) Expression of mouse *Nlrp9a/b/c* in oocytes and preimplantation embryos. The expression pattern of mouse *Nlrp9a/b/c* was analyzed using the expressed sequence tag (EST) database. TPM, transcripts per million. (C) Expression of mouse *Nlrp9a/b/c* in various adult organs and reproductive organs (insert). The expression pattern of mouse *Nlrp9a/b/c* was analyzed using RNA sequencing data of the ENCODE transcriptome project. RPKM, reads per kilobase per million reads. (D) Protein products expressed from *Nlrp9b* mutant cDNAs. Upper panel shows the structure of N-terminally, FLAG-tagged and C-terminally, Myc-tagged *Nlrp9b* carrying representative mutations. The M1 and M2 mutants carry 13 and 7 nucleotides deletions in NACHT domains, respectively, and presumably produce N-terminal truncated proteins. Calculated molecular weights are shown. The plasmids encoding wild-type (WT) and mutant *Nlrp9b* (M1, M2) were transfected into HEK293T cells. Expression of proteins were analyzed by immunoblot assay using anti-FLAG and anti-Myc antibodies (bottom panels).

The following morning (at d0.5 after mating), the oocytes and fertilized eggs were isolated from the ampulla of the oviduct. The fertilized eggs were selected according to the presence of pronuclei and the secondary polar body. The zygotes from each female were placed in individual KSOM drops and cultured at 37°C and 5% CO₂ for 4 days. Alternatively, the embryos were isolated from the uterus at d3.5 and cultured at 37°C and 5% CO₂ for 1 day. Photos of all of the embryos were taken each day using an IX70-SBF2 microscope (Olympus) and a DP73 digital charge-coupled device (CCD) camera (Olympus).

Quantification of the sizes of blastocysts and blastomeres

The images taken of the blastocysts and two-cell embryos were analyzed by ImageJ software (National Institutes of Health). All of the blastocysts were measured at d3.5 and d4.5 (Fig. 3C). Ten two-cell embryos were randomly selected for individual females, and the size of each blastomere was measured (Fig. 4E). When the number of two-cell embryos was fewer than ten, all embryos were analyzed.

Co-immunoprecipitation assay

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal calf serum and antibiotics. The plasmids encoding the FLAG-tagged Nlrp9b and Myc-tagged SCMC components were transfected using polyethyleneimine. Cell lysates were prepared in a solution of 150 mM NaCl, 1% Nonidet P-40, 10 mM Tris-HCl (pH 8.0) containing an EDTA-free protease inhibitor cocktail (Nacalai) and 10 mM MgCl₂. The Nlrp9b protein complexes were immunoprecipitated with anti-FLAG antibody-conjugated agarose beads (Sigma-Aldrich) and eluted with FLAG peptide (Sigma-Aldrich). Reciprocal coimmunoprecipitation was carried out using anti-Myc antibody (9E10 (sc-40), Santa Cruz Biotechnology). The precipitated proteins were resolved on an SDS 5–20% polyacrylamide gel (Nacalai) and transferred onto a polyvinylidene difluoride membrane (Merck Millipore). The filters were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST), and incubated sequentially with primary and secondary antibodies. The blots were developed by Chemi-Lumi One Super (Nacalai) and signals were detected using the ImageQuant LAS 4000 instrument (GE Healthcare). The antibodies used for Western blot analysis were as follows: rabbit anti-FLAG antibody (1:1,000 dilution; MBL, Aichi, Japan), rabbit anti-Myc antibody (1:500 dilution; MBL), and HRP-conjugated donkey anti-rabbit IgG F(ab')₂ fragment (1:3,000 dilution; GE Healthcare).

Immunostaining

Oocytes were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min, and permeabilized in 0.5% Triton X-100 in PBS for 15 min at room temperature. After blocking with 3% BSA in PBS for 30 min, the oocytes were incubated with primary antibodies overnight at 4°C and secondary antibody for 2 h at room temperature. The antibodies used were as follows: rabbit anti-Nlrp5 antibody (1:100 dilution), rabbit anti-Filia antibody (1:500 dilution) (Ohsugi *et al.* 2008) and CF488-conjugated goat anti-rabbit IgG (H+L) antibody (1:500 dilution; Biotium, Fremont, CA, USA). Immunofluorescence was observed using a LSM510 confocal laser scanning microscope (Carl Zeiss).

Nlrp9 antibodies

We utilized a commercially available Nlrp9 antibody (NBP2-24661, Novus Biologicals), which was used previously (Peng *et al.* 2015). We also generated antibodies using the peptides whose sequences are completely conserved among three Nlrp9 members. The peptide (KTLKLGNNNIQDT or DYLFKDFLELRNTL) were immunized individually into rabbits. The antibodies were affinity-purified using peptide columns. The ovary lysates were prepared in RIPA buffer (50 mM HEPES (pH 7.9), 150 mM NaCl, 1% Nonidet P-40, 0.5% DOC, 0.1% SDS, 5 mM EDTA, protease inhibitor cocktail) as described (Qin *et al.* 2019). Western blot analyses were performed as described.

Expression of mutant proteins

The plasmids encoding WT and mutant Nlrp9b, all of which were N-terminally, FLAG-tagged and C-terminally, Myc-tagged, were transfected into a human embryonic kidney-derived cell line, HEK293T cell. Cell lysates were prepared 48 h after transfection as described above. The protein expression was analyzed by Western blot analyses using anti-FLAG and anti-Myc antibodies as described.

Results

Generation of the Nlrp9a/b/c triple mutant (TMut) mice

The human genome contains one *NLRP9* gene, whereas the mouse genome encodes three copies of the *Nlrp9* gene: *Nlrp9a*, *Nlrp9b*, and *Nlrp9c*. Nlrp9a/b/c proteins are composed of a pyrin domain, a NACHT domain, and an LRR domain (Fig. 1A) that are almost identical; 64% of amino acids are identical among Nlrp9a/b/c. The mouse expressed sequence tag (EST) data sets showed that *Nlrp9a/b/c* expression was detected from oocytes to cleavage stage embryos, but was undetectable in blastocysts, implanted embryos and the fetus (Fig. 1B). The Nlrp9 protein was also reported to be expressed in oocytes and preimplantation embryos of mice (Peng *et al.* 2015). The RNA sequencing results of various adult organs in the mouse ENCODE transcriptome data sets showed that all three *Nlrp9* genes were expressed in ovaries (Fig. 1C) (Yue *et al.* 2014). In addition, *Nlrp9b*, but neither *Nlrp9a* nor *Nlrp9c*, was expressed strongly in digestive organs and weakly in testes. The expression of *Nlrp9a/b/c* was not detected in other adult organs. The expression pattern in adult organs was validated by quantitative RT-PCR and immunological analyses in previous studies (Peng *et al.* 2015, Zhu *et al.* 2017).

To investigate the function of *Nlrp9a/b/c* in mouse female reproduction, mutations were introduced into the *Nlrp9a/b/c* locus using the CSRI/PSR/Cas9 system. Two gRNAs were designed to target exon 2 encoding the pyrin domain of the *Nlrp9a* and *Nlrp9c* genes (*gRNAa/c*), and exon 3 encoding the NACHT domain of the *Nlrp9b* and *Nlrp9c* genes (*gRNAb/c*) (Fig. 1A). Fertilized eggs of WT mice were electroporated with *gRNAa/c* and/or *gRNAb/c* together with Cas9 protein and transferred to pseudopregnant females (Table 1). Additionally, the fertilized eggs collected from the intercrosses of the mice carrying the heterozygous mutations in both *Nlrp9a* and *Nlrp9c* were treated with *gRNAb/c*. Finally, the *Nlrp9*-mutant mice containing all genetic combinations, including single mutant (SMut; *Nlrp9a* SMut, *Nlrp9b* SMut, *Nlrp9c* SMut), double mutant (DMut; *Nlrp9a/b* DMut, *Nlrp9a/c* DMut, *Nlrp9b/c* DMut) and triple mutant (TMut; *Nlrp9a/b/c* TMut) mice, were generated (Table 1). All of the mutations resulted in premature translational termination.

To validate the absence of protein expression in ovaries of *Nlrp9* TMut females, we conducted immunoblot assays. Although three antibodies were tested, endogenous *Nlrp9* was not detected by any antibodies (data not shown; see Materials and Methods for details). As an alternative approach, we examined the expression of protein products by using N-terminally, FLAG-tagged and C-terminally, Myc-tagged *Nlrp9b* cDNAs carrying representative mutations (Fig. 1D). Following the expression vectors were transfected into HEK293T cells, the protein expression was evaluated by Western blot analyses using anti-FLAG and anti-Myc antibodies. Immunoblot analyses showed that N-terminal truncated products were barely detectable and C-terminal truncated products were undetectable. Additionally, mutations were introduced into pyrin or NACHT domains. Thus, it is plausible that functional proteins are not produced from the mutated alleles. However, as these results do not exclude the possibility that the alleles are hypomorphic, we designated these mice as mutant mice, but not knockout mice, in this study.

Although *Nlrp9* functions as inflammasome (Zhu *et al.* 2017), body weight of *Nlrp9* TMut mice was comparable to that of WT and heterozygous mice at 6–7 weeks ($P = 0.34$ by Student's *t*-test; WT, 27.9 ± 2.9 g, $n=11$; TMut, 27.9 ± 4.3 g, $n=11$) and at 6–7 months after birth ($P = 0.35$ by Student's *t*-test; heterozygous, 48.0 ± 8.7 g, $n=21$; TMut, 45.9 ± 4.6 g, $n=14$). The *Nlrp9* TMut mice did not show any abnormalities and were healthy at least for 1 year under specific pathogen-free condition.

Fertility of the *Nlrp9* TMut female mice

To examine reproductive ability, the *Nlrp9* mutant females were crossed with WT male mice for 3 months.

The number of deliveries was comparable between the WT and *Nlrp9* mutant females (Fig. 2A). In addition, litter size was not significantly different between the WT and *Nlrp9* mutant mice (Fig. 2B). However, the number of litters with seven or fewer pups was significantly increased in the *Nlrp9* TMut females compared to females with other genotypes ($P < 0.05$ by χ^2 test). Moreover, the litter size from *Nlrp9* TMut females significantly decreased compared those from all other females ($P < 0.05$ by Student's *t*-test; *Nlrp9* TMut, 8.75 ± 3.62 , $n=12$; Others, 11.24 ± 2.70 , $n=63$). We also examined when the *Nlrp9* TMut females gave birth to pups after observation of copulatory plug. The gestation period of the *Nlrp9* TMut females was slightly longer than that of WT mice ($P = 0.006$ by Student's *t*-test; WT, 19.3 ± 0.4 days, $n=6$; TMut, 20.0 ± 0.4 days, $n=7$).

The above observation prompted further examination of preimplantation development in the *Nlrp9* TMut females. The WT and *Nlrp9* TMut females were super-ovulated and crossed with WT males. At embryonic day 3.5 (d3.5), the embryos were isolated from the uterus of the females and cultured for 1 day *in vitro*. At d3.5, more than 90% of the embryos isolated from WT females developed into blastocysts (Fig. 3A and B). In contrast, the embryos isolated from the *Nlrp9* TMut females at d3.5 showed a significant decrease in the percentage of blastocysts (65%, $P < 0.0005$ by χ^2 test), as well as a significant increase in the percentages of morulae (16%, $P < 0.05$ by χ^2 test) and degenerated embryos (19%, $P < 0.005$ by χ^2 test). After 1 day of culture *in vitro*, similar trends were observed (Fig. 3A and B). Furthermore, the blastocysts were significantly smaller in the embryos from the *Nlrp9* TMut females at d3.5 and d4.5 (Fig. 3C). These results demonstrate that the blastocyst stage of development was delayed, and preimplantation embryonic death was increased in the zygotes from the *Nlrp9* TMut females *in vivo*.

Table 1 Generation of the *Nlrp9* mutant mice.

Mutants	Fertilized eggs*	gRNA	Mutations***			
			<i>Nlrp9a</i> (Pyrin)	<i>Nlrp9b</i> (NACHT)	<i>Nlrp9c</i> (Pyrin)	<i>Nlrp9c</i> (NACHT)
<i>Nlrp9a</i> SMut	WT × WT	<i>gRNAa/c</i>	+ 2nt			
<i>Nlrp9b</i> SMut	WT × WT	<i>gRNAb/c</i>		Δ 2nt		
<i>Nlrp9c</i> SMut	WT × WT	<i>gRNAb/c</i>				Δ 8nt
<i>Nlrp9a/b</i> DMut	WT × WT	<i>gRNAa/c</i> + <i>gRNAb/c</i>	+ 1nt	Δ 7nt		
<i>Nlrp9a/c</i> DMut	WT × WT	<i>gRNAa/c</i>	Δ 25nt		+1 nt	
<i>Nlrp9b/c</i> DMut	<i>Nlrp9a/c</i> hetero × <i>Nlrp9a/c</i> hetero	<i>gRNAb/c</i>		Δ 13nt		Δ 8nt
<i>Nlrp9a/b/c</i> TMut-1	<i>Nlrp9a/c</i> hetero × <i>Nlrp9a/c</i> hetero	<i>gRNAb/c</i>	Δ 25nt	Δ 13nt	+1 nt	
<i>Nlrp9a/b/c</i> TMut-2	<i>Nlrp9a/c</i> hetero × <i>Nlrp9a/c</i> hetero	<i>gRNAb/c</i>	Δ 25nt	Δ 13nt	+1 nt **	Δ 1nt

*The fertilized eggs from the WT (WT) females that were crossed with WT males or from the *Nlrp9a/c* heterozygous mutant females that were crossed with the *Nlrp9a/c* heterozygous mutant males were treated with *gRNAa/c* and/or *gRNAb/c*. **The insertion of 1 nucleotide in Pyrin domain resulted in premature translational termination before the position of the 1 nucleotide deletion in NACHT domain. Therefore, the same truncated *Nlrp9c* proteins are produced both in the *Nlrp9a/b/c* TMut-1 and the *Nlrp9a/b/c* TMut -2 mice. ***All the mutations resulted in premature translational termination immediately after in/del mutation sites.

Figure 2 Reproductive ability of the *Nlrp9* mutant female mice. The number of deliveries per three months (A) and the number of pups per delivery (B). The wild-type (WT) females and the *Nlrp9* mutant females carrying single-, double-, and triple- mutations (SMut, DMut, and TMut, respectively) were crossed with wild-type males. The number of deliveries and litter size were not significantly changed in any of the *Nlrp9* mutant mice (Student's *t*-test). The mean $\pm$ standard deviation is shown. The numbers of mice examined were as follows; wild-type, $n = 3$; *Nlrp9a* SMut $n = 4$; *Nlrp9b* SMut $n = 4$; *Nlrp9c* SMut $n = 2$; *Nlrp9a/b* DMut $n = 3$; *Nlrp9a/c* DMut $n = 4$; *Nlrp9b/c* DMut $n = 3$; *Nlrp9a/b/c* TMut $n = 5$.

Development of zygotes from the *Nlrp9* TMut female mice *in vitro*

To further explore the roles of *Nlrp9a/b/c* in preimplantation development, fertilized eggs isolated from oviducts of *Nlrp9* TMut females were analyzed *in vitro*. Wild-type, *Nlrp9a/b/c* heterozygous, and *Nlrp9* TMut females were super-ovulated and crossed with WT males. The fertilized eggs were isolated from the oviducts at d0.5. The number of ovulated eggs was comparable between the control (wild-type and *Nlrp9a/b/c* heterozygous) females and *Nlrp9* TMut females ($P = 0.40$ by Student's *t*-test; control, 20.3 ± 13.9 , $n = 9$; *Nlrp9* TMut, 27.2 ± 15.1 , $n = 6$). Additionally, the fertilization rate was not significantly altered between the control and the *Nlrp9* TMut females ($P = 0.07$ by Student's *t*-test; control, $74.9\% \pm 15.8\%$, $n = 9$; *Nlrp9* TMut, $92.9\% \pm 17.4\%$, $n = 6$).

The fertilized eggs were then cultured for 4 days *in vitro*. The zygotes isolated from control females developed progressively into blastocysts over the 4 days (Fig. 4A and B). Approximately 70% of the embryos from control females developed into blastocysts at d4.5 (Fig. 4C). In contrast, in the zygotes from the *Nlrp9*

Figure 3 Development of preimplantation embryos in the *Nlrp9* TMut females *in vivo*. (A) Representative photos of the embryos at embryonic day 3.5 (d3.5) and d4.5. The embryos were isolated from the uterus of wild-type and *Nlrp9* TMut females at d3.5 after mating with wild-type males (d3.5, left), and cultured for 1 day *in vitro* (d4.5, right). Note that the blastocysts from the *Nlrp9* TMut females were smaller in size. Morulae and the degenerated embryos were also observed in the embryos from the *Nlrp9* TMut females. Bars: 50 μ m. (B) The percentages of blastocysts, morulae, and degenerated embryos. The number of blastocysts at d3.5 was significantly lower in the embryos isolated from *Nlrp9* TMut females ($P < 0.0005$ by χ^2 test). The number of morulae and degenerated embryos at d3.5 significantly increased in the embryos of *Nlrp9* TMut females ($P < 0.05$, $P < 0.005$ by χ^2 test, respectively). The number of blastocysts decreased, and the number of degenerated embryos increased, in the *Nlrp9* TMut females at d4.5 ($P < 0.001$ by χ^2 test). Wild-type, 57 embryos from 4 females; *Nlrp9* TMut, 86 embryos from 5 females. (C) Relative sizes of blastocysts. The box plots show the 25th, 50th, and 75th percentiles of blastocyst size and the whiskers show the minimum and maximum values. The blastocysts isolated from the *Nlrp9* TMut females were significantly smaller (Student's *t*-test; $*P < 10^{-7}$, $**P < 0.05$). Wild-type, 53, and 56 blastocysts at d3.5 and d4.5, respectively; *Nlrp9* TMut, 56, and 68 blastocysts at d3.5 and d4.5, respectively.

TMut females, although ~60% of the zygotes developed into two-cell embryos at d1.5, the remaining zygotes did not proceed to the two-cell stage and underwent degeneration. Furthermore, the majority of the two-cell embryos were arrested at this stage, and no embryos reached the blastocyst stage until d4.5 (Fig. 4A, B and C).

Asymmetric cell division, as well as the two-cell block, are commonly observed in zygotes from females lacking SCMC components (Yu *et al.* 2014, Gao *et al.* 2018). We analyzed the sizes of two blastomeres in the two-cell

Figure 4 Development of zygotes from the *Nlrp9* TMut females *in vitro*. (A) Representative photos of the embryos developed *in vitro*. The fertilized eggs were isolated at d0.5 from control (wild-type and the *Nlrp9a/b/c* heterozygous) females and *Nlrp9* TMut females that were crossed with wild-type male mice, and cultured from d0.5 to d4.5 *in vitro*. Note that the degenerated embryos emerged at d1.5, and the majority of the embryos were arrested at the 2-cell stage in the *Nlrp9* TMut females. Bar: 50 μ m. (B) Development from 1-cell embryos to blastocysts *in vitro*. Control, 86 embryos from 4 females; *Nlrp9* TMut, 148 embryos from 6 females. (C) Percentages of zygotes developed into blastocysts *in vitro*. The fertilized eggs were isolated from control and *Nlrp9* TMut females ($n=4$ and $n=6$, respectively), and the percentage of the blastocysts at d4.5 for each mouse is plotted. No zygotes developed into blastocysts in the *Nlrp9* TMut females (Student's *t*-test; $*P < 10^{-4}$). The mean $\pm$ S.D. is shown. (D) Representative photos of the two-cell embryos with asymmetric cell division from *Nlrp9* TMut females. Bars: 20 μ m. (E) The percentage of the two-cell embryos with asymmetric cell division. The embryos were classified as undergoing asymmetric division when the sizes of two blastomeres differed by more than 10%. The percentage of embryos with asymmetric cell division was significantly higher in the zygotes from *Nlrp9* TMut females (Student's *t*-test; $*P < 10^{-3}$).

embryos at d1.5 (Fig. 4D). The two-cell embryos were classified as those with asymmetric cell division when the sizes of two blastomeres differed by more than 10%. The percentage of two-cell embryos with asymmetric cell division was significantly elevated in embryos from the *Nlrp9* TMut females (Fig. 4E).

Development of the zygotes from the *Nlrp9* DMut female mice *in vitro*

To examine the contribution of each *Nlrp9* family member to the two-cell block observed in the zygotes from the *Nlrp9* TMut females *in vitro*, the fertilized eggs isolated from the *Nlrp9* DMut females were cultured for four days *in vitro*. The zygotes isolated from *Nlrp9a/b* DMut, *Nlrp9a/c* DMut and *Nlrp9b/c* DMut females did not exhibit two-cell block (Fig. 5). However, the embryos from these DMut mice displayed developmental delay and arrest. For example, the zygotes from *Nlrp9a/b* DMut and *Nlrp9a/c* DMut females showed delayed development to blastocysts at d3.5, although the majority developed to blastocysts at d4.5 (Fig. 5A and B). In the zygotes from *Nlrp9b/c* DMut females, more than half of the embryos remained at the 4–8-cell stages, ~40% were morulae, and no blastocysts were observed at d3.5 (Fig. 5A and B). Moreover, at d4.5, only 40% of embryos developed to blastocysts, whereas ~40% of embryos were degenerated. Furthermore, the blastocysts were significantly smaller in the embryos from the *Nlrp9a/b* DMut, *Nlrp9a/c* DMut and *Nlrp9b/c* DMut females at d4.5 (Fig. 5C). These results demonstrated that maternal *Nlrp9a/b/c* are redundantly required for development beyond the two-cell stage *in vitro*.

Physical associations between *Nlrp9* and the SCMC components

Because two-cell block and asymmetric cell division are characteristic of females lacking the SCMC components, *Nlrp9* may participate in the formation of the SCMC. We examined whether *Nlrp9* can bind to major SCMC components. After the expression vector for FLAG-tagged *Nlrp9b* was transfected into HEK293T cells with plasmids encoding Myc-tagged SCMC components, including *Nlrp5* (MATER), *Filia* or *Floped* (Ooep), FLAG-*Nlrp9b* was pulled down with anti-FLAG antibody-agarose. As shown in Fig. 6A, all these SCMC components were coimmunoprecipitated with *Nlrp9b*. In addition, reciprocal coimmunoprecipitation analyses showed that *Nlrp9b* was coprecipitated with all these SCMC components (Fig. 6B). These results suggest the possibility that *Nlrp9* is a novel component of the SCMC.

The subcortical distribution of SCMC components are disturbed in the oocytes lacking the core components of SCMC, including *Nlrp5* and *Ooep* (Li *et al.* 2008, Yu *et al.* 2014, Mahadevan *et al.* 2017). Therefore, we investigated whether the subcellular localization

of *Nlrp5* and *Filia* was altered by the mutations of *Nlrp9a/b/c*. Similar to control mice, *Nlrp5* and *Filia* was localized in subcortical regions in oocytes of *Nlrp9* TMut mice (Fig. 6C), suggesting that *Nlrp9a/b/c* is not required for subcortical localization of SCMC.

Discussion

In this study, we analyzed the developmental potential of oocytes lacking maternal *Nlrp9a/b/c* in mice. The zygotes developed in the *Nlrp9* TMut females *in vivo* exhibited increased lethality and a delay in reaching the blastocyst stage (Fig. 3). Furthermore, the fertilized eggs isolated from the *Nlrp9* TMut females displayed two-cell block in an *in vitro* culture, which was accompanied by asymmetric cell division (Fig. 4). These results demonstrated that *Nlrp9a/b/c* contribute to optimal progression of preimplantation development. On the other hand, zygotes from three types of *Nlrp9* DMut females did not show two-cell block, although the development of these zygotes was delayed or arrested around the 4–8-cell stages *in vitro* (Fig. 5). Our results showed that the presence of any one of *Nlrp9a/b/c* could rescue the two-cell block in *Nlrp9* TMut mice, indicating that *Nlrp9a/b/c* are functionally redundant in preimplantation development.

The two-cell block and asymmetric cell division observed in the zygotes of the *Nlrp9* TMut females are reminiscent of the zygotes of the female mice lacking the components of the SCMC (Yu *et al.* 2014, Gao *et al.* 2018). It was shown previously that the SCMC is a multiprotein complex composed of *Nlrp5*, *Floped*, *Filia*, and *Tle6* (Li *et al.* 2008). However, considering that the estimated molecular weight of the SCMC ranged from 669 kDa to 2 MDa (Li *et al.* 2008), it is likely that the SCMC contains additional components. Recent reports have revealed that *Nlrp2* and *Nlrp4f*, other members of the reproductive NLRPs, and *Zbed3*, are components of the SCMC (Mahadevan *et al.* 2017, Gao *et al.* 2018, Qin *et al.* 2019). Our study has also shown that *Nlrp9b* was able to bind to the SCMC components (Fig. 6A and B). Taken together, while *Nlrp9b* forms an inflammasome composed of adaptor protein PYCARD (ASC), which recruits caspase-1, and RNA helicase *Dhx9*, which binds to rotavirus RNA in the intestinal epithelium (Zhu *et al.* 2017), *Nlrp9a/b/c* may participate in the SCMC as novel components in oocytes and preimplantation embryos. On the other hand, while subcortical localization of SCMC components was compromised by the absence of 'core' components of SCMC such as *Nlrp5*, *Ooep* and *Tle6* (Li *et al.* 2008, Yu *et al.* 2014, Mahadevan *et al.* 2017), the distribution of the SCMC components was not altered in the oocytes lacking *Filia*, *Zbed3* and *Nlrp4f* (Zheng & Dean 2009, Gao *et al.* 2018, Qin *et al.* 2019). Normal distribution of *Nlrp5* and *Filia* in *Nlrp9* TMut oocytes suggests that *Nlrp9a/b/c* are not the core component (Fig. 6C).

Figure 5 Development of zygotes from the *Nlrp9* DMut females *in vitro*. (A) Representative photos of the embryos developed *in vitro*. Fertilized eggs were isolated at d0.5 from control and *Nlrp9* DMut females that were crossed with wild-type male mice, and cultured from d0.5 to d4.5 *in vitro*. Bar: 50 μ m. (B) Development from one-cell embryos into blastocysts *in vitro*. Blastocyst development was significantly delayed at d3.5 in the embryos from *Nlrp9a/b* DMut and *Nlrp9a/c* DMut females ($P < 10^{-8}$ and $P < 10^{-9}$ by χ^2 test, respectively). Development into blastocysts significantly decreased, and the incidence of degeneration increased, at d4.5 in *Nlrp9b/c* DMut females ($P < 10^{-13}$ and $P < 10^{-8}$, respectively, by χ^2 test). Wild-type, 128 embryos from 9 females; *Nlrp9a/b* DMut, 86 embryos from 5 females; *Nlrp9a/c* DMut, 103 embryos from 5 females; *Nlrp9b/c* DMut, 131 embryos from 5 females. (C) Relative sizes of blastocysts at d4.5. The box plots show the 25th, 50th, and 75th percentiles of blastocyst size and the whiskers show the minimum and maximum values. The blastocysts isolated from three types of *Nlrp9* DMut females were significantly smaller (Student's *t*-test; * $P < 10^{-11}$, ** $P < 10^{-12}$, *** $P < 10^{-18}$). Wild-type, $n=73$; *Nlrp9a/b* DMut, $n=74$; *Nlrp9a/c* DMut, $n=78$; *Nlrp9b/c* DMut, $n=56$.

It is well-known that under *in vitro* culture conditions, fertilized eggs from permissive mouse strains can develop into blastocysts, whereas those from non-permissive strains are arrested at the two-cell stage (Biggers 1998). As females of non-permissive strains give birth to offspring during natural mating, the suboptimal conditions in culture induce the two-cell block, which is dependent on the genetic background

of mice. Similar to the permissive mouse strains, the *Nlrp9* TMut females in this study gave birth to a relatively normal number of pups, but the zygotes from the *Nlrp9* TMut females exhibited two-cell block in culture. Likewise, the zygotes from the *Nlrp2* mutant females also resulted in two-cell block in culture, although the number of implanted embryos in the mutant females *in vivo* was comparable to those of the

Figure 6 Physical association between Nlrp9 and the SCMC components. (A) The FLAG-Nlrp9b-associated proteins were isolated with anti-FLAG antibody-conjugated agarose from the HEK293T cell lysates that were transfected with the indicated plasmids, and then analyzed by immunoblot assay with anti-Myc and anti-FLAG antibodies (left panel). The right panel shows the input cell lysates analyzed by immunoblot assay with anti-Myc and anti-FLAG antibodies. FLAG-Nlrp9b could bind to all the Myc-tagged SCMC components, including Filia, Ooep, and Nlrp5. (B) Reciprocal coimmunoprecipitation analyses using anti-Myc antibody. (C) Subcortical distribution of the SCMC components, Nlrp5 and Filia, in oocytes from the *Nlrp9* TMut females. Oocytes were analyzed using a confocal laser scanning microscope with 1- μ m-thick optical sections.

control females (Mahadevan *et al.* 2017). Additionally, the zygotes from *Nlrp4f* KO females did not exhibit two-cell arrest, but showed delayed development to blastocysts *in vitro* (Qin *et al.* 2019). It has been shown that the formation of the F-actin meshwork around the subcortical region was impaired in the absence of the

SCMC components, which may have given rise to the asymmetric cell division seen in the SCMC mutant embryos (Yu *et al.* 2014). Thus, the molecular processes in which reproductive NLRPs are involved, including the dynamics of cytoskeletal organization, may be affected by the culture conditions.

Female mice deficient in *Nlrp5* are sterile due to the two-cell block *in vivo* (Tong *et al.* 2000). The *Nlrp2* mutant female mice frequently give birth to stillborn offspring with aberrant DNA methylation in the imprinted genes (Mahadevan *et al.* 2017). In this study, we showed that the *Nlrp9* TMut female mice had relatively normal litter sizes, but preimplantation development was impaired both *in vitro* and *in vivo*. As they lacked one member of the reproductive NLRPs, this resulted in specific phenotypes, suggesting that reproductive NLRPs might be functionally diverse. Each immune NLRP forms an independent inflammasome that responds to distinct pathogens and stressors. Similarly, reproductive NLRPs might also be involved in the formation of diverse SCMCs. As an alternative, the milder phenotypes of the *Nlrp2* KO, *Nlrp4f* KO and *Nlrp9* mutant mice might be explained by compensation by *Nlrp5*. In conclusion, taken together with a previous study (Zhu *et al.* 2017), our results demonstrated that Nlrp9 functions as both an immune and reproductive NLRP, depending on the cell types involved.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Sa K, S T, T I, M W and Y S performed the experiments. All the authors analyzed the data. Sa K, S T, Y S and T K designed the research and wrote the paper. All the authors approved the manuscript.

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