**Nlrp2**, a Maternal Effect Gene Required for Early Embryonic Development in the Mouse

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Abstract

Maternal effect genes encode proteins that are produced during oogenesis and play an essential role during early embryogenesis. Genetic ablation of such genes in oocytes can result in female subfertility or infertility. Here we report a newly identified maternal effect gene, Nlrp2, which plays a role in early embryogenesis in the mouse. Nlrp2 mRNAs and their proteins (~118 KDa) are expressed in oocytes and granulosa cells during folliculogenesis. The transcripts show a striking decline in early preimplantation embryos before zygotic genome activation, but the proteins remain present through to the blastocyst stage. Immunogold electron microscopy revealed that the NLRP2 protein is located in the cytoplasm, nucleus and close to nuclear pores in the oocytes, as well as in the surrounding granulosa cells. Using RNA interference, we knocked down Nlrp2 transcription specifically in mouse germinal vesicle oocytes. The knockdown oocytes could progress through the metaphase of meiosis I and emit the first polar body. However, the development of parthenogenetic embryos derived from Nlrp2 knockdown oocytes mainly blocked at the 2-cell stage. The maternal depletion of Nlrp2 in zygotes led to early embryonic arrest. In addition, overexpression of Nlrp2 in zygotes appears to lead to normal development, but increases blastomere apoptosis in blastocysts. These results provide the first evidence that Nlrp2 is a member of the mammalian maternal effect genes and required for early embryonic development in the mouse.

Introduction

Maternal factors, which are encoded by maternal effect genes and accumulate during oogenesis, are critical to support the development of the preimplantation embryo [1]. Maternal effect genes were first described in mammals in 2000 [2,3], although some early pioneering investigations had been reported in *Drosophila* [4,5], *Caenorhabditis elegans* [6] and *Xenopus* [7]. Several maternal effect genes that function in oogenesis [8], meiotic maturation [9], preimplantation [8,10,11,12,13,14,15,16] and postimplantation development [17] have been identified in the mouse. However, knowledge of the maternal effect genes involved in female reproduction and early embryo development is still limited in mammals.

NLRP (Nucleotide-binding oligomerization domain, Leucine-rich Repeat and Pyrin domain containing Proteins) is a subfamily of the newly described CATERPILLER (CASpase-recruitment domain (CARD) Transcription Enhancer, R (purine)-binding, Pyrin, Lots of LEucine Repeats) family of proteins with a nucleotide-binding domain and a leucine-rich region [18,19]. The Nlp genes contain 20 members in the mouse. Recent studies have demonstrated that these genes play key roles in reproduction. For instance, the mouse *Nlp5* gene, known to encode for the Maternal Antigen That Embryos Require (MATER) [20], was one of the earliest maternal effect genes characterized at the molecular level in the mouse. Although showing fairly normal folliculogenesis, oocyte maturation and fertilization, *Nlrp5* knockout female mice are infertile because of a block of embryogenesis at the 2-cell stage [3]. Recently, a subcortical maternal complex (SCMC), constituting MATER, FLOPED, TLE6 and FILIA has been identified. This assembles during oocyte growth, [15,21]. It is essential for zygotes to progress beyond the first embryonic cell divisions. In vitro knockdown of *Nlrp14* in mouse zygotes leads to developmental arrest between the 2-cell and the 8-cell stages in more than 50% of the embryos [22]. Besides *Nlrp5* and *Nlrp14*, several other *Nlp* genes also display specific or preferential oocyte expression patterns, the expression of which declines with oocyte aging [22,23]. Additionally, in studies on the oocyte-to-embryo transition in mouse, *Nlrp2* (also known as *Nalp2/Pan1/Pypaf2*) was shown to be highly enriched in fully grown oocytes by reverse transcription polymerase chain reaction (RT–PCR) amplification [24]. Based on these limited previous studies, we hypothesized that *Nlrp2* would be essential for early embryo development and that perturbing its function in the oocyte and zygote would disrupt early embryogenesis.

To test this hypothesis, we employed an RNA interference (RNAi) approach to inhibit *Nlrp2* function specifically in mouse germinal vesicle (GV) oocytes and zygotes. This is an efficient method for inhibiting the function of maternal and zygotically expressed genes in oocytes and zygotes [25,26,27,28]. Electropo-
ration was used to deliver silencing RNA because it is one of the most effective ways of delivering double stranded (ds)RNA and small interfering (si)RNA into mouse oocytes and early embryos [29,30]. In addition, we constructed an expression vector for Nlrp2 and microinjected it into mouse zygotes.

We report here that the maternal depletion of Nlrp2 in GV-stage oocytes and zygotes results in early developmental arrest. However, overexpression of Nlrp2 in zygotes appears to lead to normal development, but increases apoptosis in blastocyst. In addition, Nlrp2 is not required for oocyte maturation. Thus, Nlrp2 can be added to the increasing—if limited—list of mammalian maternal effect genes.

**Results**

*Nlrp2* mRNA Expression Levels

*Nlrp2* expression was detected in mouse ovaries, but not in eleven other tissues, including male testes (Fig. 1A). Within the ovary, expression was restricted to ovarian follicles at various stages (Fig. 1B). After ovulation and fertilization, the transcripts can be added to the increasing—if limited—list of mammalian maternal effect genes.

\[ \text{Fig. 1. Developmental expression of Nlrp2 in the mouse.} \]

(A) Quantitative reverse-transcription polymerase chain reaction (qRT–PCR) with total RNA extracted from 4-week-old mouse ovary (Ov), uterus (Ut), testis (Te), kidney (Ki), lung (Lu), heart (He), liver (Li), brain (Br), stomach (St), intestines (In), muscle (Mu), spleen (Sp) were performed. Results were normalized to the abundance in the ovary and expressed as the mean ± SEM.

(B) In situ hybridization of fixed, paraffin wax-embedded 6 µm ovary sections probed with DIG-labeled Nlrp2 oligonucleotide probes. The original magnification was ×100.

(C) The relative abundance of Nlrp2 transcripts in mouse oocytes and preimplantation embryos. (D) The relative abundance of Nlrp2 transcripts in different mouse cells.

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were immediately downregulated and not detected after the 2-cell stage during preimplantation development (Fig. 1C). In addition, except for oocytes and cumulus cells, Nlrp2 mRNA was not detected in other cell lines (Fig. 1D), although human NLRP2 is expressed in various tumor lines [31].

NLRP2 Protein Levels

Immunohistochemistry, immunofluorescence and immunoblotting were used to assess expression at the protein level. Immunohistochemistry of ovarian sections suggested that the NLRP2 protein was expressed in oocytes and granulosa cells at various follicular stages (Fig. 2A). After ovulation and fertilization, the protein (~118 KDa) was still present in cumulus–oocyte complexes (Fig. 2B) and remained present through the blastocyst stage (Fig. 2C) although Nlrp2 mRNA was not detected after the 2-cell stage (Fig. 1C).

Cellular and Subcellular Localization of NLRP2

To determine the location of NLRP2 protein in oocytes and early embryos, we conducted confocal and immunogold electron microscopy analyses. Confocal microscopy demonstrated a predominant cytoplasmic location of the NLRP2 protein in oocytes and early embryos (Fig. 3). The distribution of NLRP2 protein in the subcellular organelles was monitored by immunogold staining and transmission electron microscopy. Immunogold particles were seen within the oocytes and the surrounding granulosa cells. They were mainly located in the cytoplasm, but some particles were present in the nucleus and close to nuclear pores in the oocytes (Fig. 4A), as well as the surrounding granulosa cells (Fig. 4B).

Expression and Localization of Nlrp2 in Parthenogenetic Embryos

The amount of Nhp2 mRNA declined sharply after parthenogenetic activation of metaphase II oocytes (Fig. 5A). As the parthenogenetic embryos developed beyond the 2-cell stage, the transcripts virtually disappeared (Fig. 5A). However, the NLRP2 protein remained present at preimplantation stages (Fig. 5B). The protein was predominately localized in the blastomere cytoplasm (Fig. 5C). Thus, the expression profile of NLRP2 protein during embryogenesis, as determined by immunoblotting assay, was similar to that determined by confocal microscopic analysis.

Nlrp2 Is Not Required for Oocyte Maturation

Zona-weakened germinal vesicle (GV)-stage oocytes were electroporated with custom-made Nlrp2 siRNA mixture. As shown in Figure 6A, Nlrp2 siRNA had no obvious effect on oocyte maturation. However, the Nhp2 gene was downregulated in a dose-dependent manner (Fig. 6B). The Nhp2 knockdown effects were specific as the expressions of Nlp-1f, Nlp-5, Nlp-9c and Nlp-14, randomly selected members of the Nlp gene family associated with reproduction and embryo development, were not altered by the

Figure 2. Developmental expression of NLRP2 protein in mouse. (A) Immunohistochemical analysis of sequential sections from a 3-week-old mouse ovary using an anti-NLRP2 antibody. The original magnification was ×40. (B) Immunofluorescent detection of NLRP2 in cumulus–oocyte complexes after permeabilization and incubation with an anti-NLRP2 antibody. The original magnification was ×100. (C) Immunoblots of lysates isolated from oocytes and preimplantation embryos. Molecular masses (KDa) are indicated on the left; β-actin was used as a control. doi:10.1371/journal.pone.0030344.g002
introduction of Nlrp2 siRNA (60 nM) (Fig. 6C). Moreover, analysis of NLRP2 protein levels by immunoblotting showed that this obviously decreased in oocytes 24 h after electroporation with Nlrp2 siRNA compared with control groups (Fig. 6D). Thus, the decline of Nlrp2 mRNA and protein in oocytes after electroporation with Nlrp2 siRNA did not affect oocyte maturation.

Nlrp2 Knockdown in Oocytes Leads to Early Embryonic Arrest After Parthenogenetic Activation

Metaphase II oocytes (after extrusion of the first polar body) derived from oocytes electroporated at the GV stage were subjected to parthenogenetic activation and then cultured in KSOMaa-BSA medium (see Materials and Methods). There was no significant difference in the parthenogenetic activation rate of the oocytes obtained from the EP control, control siRNA or Nlrp2 siRNA (20, 40 and 60 nM) groups (Fig. 7A). The embryonic developmental stages and their morphological appearance after being cultured for 3.5 days are presented in Figure 7B. The parthenogenetic embryos derived from the electroporation (EP) control and control siRNA reached the blastocyst stage at rates of 65% and 63%, respectively (Fig. 7C). However, the development of parthenogenetic embryos derived from Nlrp2 siRNA-electroporated oocytes appeared to be blocked mainly at the 2-cell stage (Fig. 7C).

Nlrp2 Knockdown in Zygotes Causes Early Embryonic Arrest

To further confirm the function of Nlrp2 in early embryonic development, the zygotes were electroporated with Nlrp2 siRNA. As shown in Figure 8A, the introduction of Nlrp2 siRNA resulted in the reduction of the targeted mRNA in time- and concentration-dependent manners. Moreover, it also led to a decrease in the NLRP2 protein level compared with control groups (Fig. 8B). Morphological analysis suggested that embryos derived from Nlrp2 siRNA-electroporated zygotes appeared to be arrested, while the embryos derived from control groups reached the blastocyst stage (Fig. 9). The Nlrp2 knockdown embryos were mainly arrested between the 2- and 8-cell stages (Fig. 10).

Overexpression of Nlrp2 in Zygotes Leads to Normal Development but Increased Apoptosis

As described above, the knockdown of Nlrp2 with target-specific siRNA in oocytes or zygotes led to early developmental arrest. However, the effect of overexpression in zygotes is unknown. To determine this, zygotes were microinjected with pIRES2-AcGFP1-Nuc-Nlrp2 (pIRES2-Nlrp2), a bicistronic vector independently expressing NLRP2 and green fluorescent protein (GFP) (see Materials and Methods). Zygotes microinjected with pIRES2-Nlrp2 or pIRES2-AcGFP1-Nuc (pIRES2) reached the blastocyst stage and expressed GFP (Fig. 11A). The rates of blastocyst formation were similar between the two groups (Fig. 11B). As shown in Figure 11C, the Nlrp2 transcripts were reduced dramatically and were undetectable after the 2-cell stage in the control group. However, the expression of Nlrp2 persisted during preimplantation development in the group microinjected with pIRES2-Nlrp2. As expected, the level of NLRP2 protein in the pIRES2-Nlrp2-microinjected group gradually increased during preimplantation stages compared with the control group (Fig. 11D). To confirm whether accumulation of NLRP2 protein would influence cell fate and differentiation in blastocysts, we investigated the expression levels of OCT3/4 and CDX2, markers of pluripotency and trophectoderm differentiation [32,33,34,35], respectively. The final cell fate and differentiation was not affected with regard to the expression of these markers (Fig. 12A). We also examined whether accumulation of NLRP2 protein would lead to apoptosis in blastocysts. We found that the number of apoptotic blastomeres in the pIRES2-Nlrp2 microinjected group was significantly increased compared with the control group (Fig. 12B, C).
The Nlrp gene family plays a pivotal role in the innate immune and reproductive systems in mammals. Nlrp5 (Mater) and Nlrp14 are members of this family and are required for early embryonic development [3,22]. We report here that Nlrp2, another member of this family, is a newly identified maternal effect gene. It is essential for female fertility, because developmental competence is dramatically compromised in Nlrp2 knockdown oocytes and zygotes. Our data demonstrate that maternal depletion of Nlrp2 blocks early embryogenesis in the mouse.

Maternal effect genes encode proteins that are produced during oogenesis and play a role during early embryogenesis. We demonstrated here that the temporal and spatial patterns of Nlrp2 expression were developmentally regulated in a manner typical of maternal effect genes. Nlrp2 mRNA appeared to accumulate during oogenesis and degrade after zygotic genome activation. Hence, the source of embryonic Nlrp2 transcripts is maternal and does not come from expression of the zygotic genome during preimplantation development. NLRP2 proteins were detected at different stages of follicle development, suggesting that the Nlrp2 gene is involved in transcription-translation coupling and might play a role during folliculogenesis. After the 2-cell stage in

Figure 4. Subcellular localization of NLRP2 protein. (A) Subcellular localization of NLRP2 protein in immature mouse oocytes. Using an anti-NLRP2 antibody and ultrathin ovarian sections of 10-day-old mice, immunogold reactions were examined by transmission electron microscopy. Black spots with arrows are immunogold particles indicating the presence of NLRP2 protein. a, Oocytes and surrounding granulosa cells (×4,000). The positions of nucleus (i), cytoplasm (ii) and granulosa cells (iii) are indicated. b and c, Oocyte cytoplasm with immunogold particles (×50,000). d, Nucleus with immunogold particles (×50,000). e, Nuclear pore with immunogold particles nearby (×50,000). f, Control oocyte without immunogold particles in the absence of the primary antibody (×50,000). (B) Subcellular localization of NLRP2 protein in mouse granulosa cells. a, Granulosa cell (×15,000). The positions of the nucleus (i) and cytoplasm (ii). b and c, Cytoplasm with immunogold particles (×50,000). d, Nucleus with immunogold particles (×50,000). e, Nucleus and nuclear pore with immunogold particles (×50,000). f, Granulosa cell without immunogold particles in the absence of primary antibody (×50,000).

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embryos, Nlrp2 mRNA was undetectable, but the protein persisted through to the blastocyst stage. The expression patterns of Nlrp2 mRNA and its protein in parthenogenetic embryos were identical to those in normally fertilized embryos, suggesting that Nlrp2 might be involved in two different developmental processes, but fulfill the same function.

The NLRP2 protein was distributed predominantly in the cytoplasm both in normal fertilized embryos and parthenogenetic embryos as shown by confocal microscopy. Immunogold electron microscopy demonstrated that the protein was present in the cytoplasm and close to nuclear pore complexes. In addition, immunogold particles were detected within the nucleus. However, the NLRP2 sequence does not display a nuclear localization signal. It is likely that other proteins interacting with NLRP2 mediate its transport into the nucleus; indeed, this protein contains leucine-rich repeat domains, which are known to participate in protein–protein interactions [49,50,51]. The subcellular localization of NLRP2 protein differs from NLRP5 (MATER) in mouse [52] and bovine oocytes [53]. Such localization of NLRP2 in multiple organelles suggests its participation in different intracellular functions.

Mouse embryos with mutations in maternal effect genes are characterized by developmental arrest during oogenesis or embryogenesis. Among the known maternal effect genes, only Formin-2−/− and basonaucin-deficient oocytes cannot undergo normal maturation [8,9]. Compared with Formin-2 null and basonaucin-deficient oocytes, Nlrp2 knockdown GV-stage oocytes could reach metaphase II and emit the first polar body, suggesting that Nlrp2 is not required for the metaphase spindle positioning or migration. Moreover, Nlrp2 deficiency in oocytes does not perturb RNA polymerase I- or -II-mediated transcription, as the disruption of these processes leads to abnormal oocyte maturation [8,9,54]. However, the parthenogenetic embryos derived from Nlrp2 knockdown oocytes were mainly arrested at the 2-cell stage. Interestingly, the expression levels of Nlrp4f, Nlrp5, Nlrp9c and Nlrp14 were not affected in Nlrp2 knockdown oocytes, suggesting that the expression of other Nlrp genes was not able to compensate for the absence of Nlrp2. Furthermore, the predominant 2- and 8-
cell stage arrest of embryos derived from Nlrp2 knockdown zygotes suggests that this maternal factor functions in early embryonic development. Previous studies also indicated that targeted invalidation/depletion of Nlrp5 and Nlrp14 does not reveal any gene compensation. In addition, microinjection with the pIRES2-Nlrp2 vector into zygotes did not affect subsequent cell fate and differentiation in terms of the expression and localization of OCT3/4 or CDX2. However, overexpression of Nlrp2 increased the incidence of apoptosis in blastocysts, implying that an excessive accumulation of NLRP2 protein might impair the viability of blastomeres or activate the pathway of cell apoptosis, because NLRP2 contains a pyridine domain at the amino terminal that is primarily involved in apoptosis [55,56,57].

There are several reproduction-related Nlrp genes with conserved functions in mammals and primates. Nlrp5 (NLRP5) transcripts are detected in the ovary in the mouse [3,20], bovine [53,58,59], pig [60], rhesus macaque monkeys [28,48] and humans [61]. Moreover it has been demonstrated that Nlrp5 plays key roles in early embryo development in the mouse [3] and rhesus macaque monkeys [28]. Nlrp14 is continuously expressing in germ cells [62] and in vitro knockdown experiments in zygotes led to developmental arrest in the mouse [22]. Moreover mutations in the testis-specific NLRP14 gene in men leads to spermatogenic failure [63], implying that these genes are conserved functionally in a wide range of mammalian reproductive systems. Germline mutations in NLRP2 result in a familial imprinting disorder (Beckwith–Wiedemann Syndrome) in humans [64], suggesting that this gene might have a similar function in the establishment and/or maintenance of genomic imprinting/methylation in the mouse. The Dmutlo mutation causes disrupted genomic imprinting in early mouse embryogenesis [17]. Moreover, a gene disruption study determined that PGC7/Stella is indispensable for the maintenance of methylation involved in epigenetic reprogramming after fertilization [11,65,66]. Thus, the function of Nlrp2 might be similar to the maternal effect gene Dmutlo and/or PGC7/Stella during early preimplantation development. However, the
Localization of Nlrp2 in the preimplantation embryo is very different, so the molecular function of Nlrp2 may be different from Dnmt1o and PGC7/Stella. Identification of proteins that interact with NLRP2 could provide insights into the roles of this maternal factor in regulating the critical stages of development from the oocyte to the preimplantation embryo.

In summary, our data demonstrate that Nlrp2 is a maternal effect gene and required for early embryonic development in the mouse. The maternal depletion of Nlrp2 in zygotes by RNAi results in early embryonic arrest. Nlrp2 knockdown oocytes can progress through the metaphase of meiosis I and emit the first polar body; however, the development of parthenogenetic embryos derived from Nlrp2 knockdown oocytes mainly arrest at the 2-cell stage. In addition, overexpression of Nlrp2 in zygotes appears to permit normal development, but increases the incidence of apoptosis in blastocysts.

Materials and Methods

Ethics Statement

The experimental procedure was approved by the Animal Care Commission of the College of Veterinary Medicine, Northwest A&F University. Adult male and female ICR strain mice were

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Figure 7. Development of parthenogenetic embryos derived from EP control, control siRNA and Nlrp2 siRNA-treated groups (20, 40 and 60 nM). (A) Parthenogenetic activation rate of electroporated oocytes. (B) Morphological appearance of parthenogenetic embryos after being cultured for 3.5 days. The original magnification was ×100. (C) Percentage of parthenogenetic embryos at different stages after being cultured for 3.5 days.

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purchased from the Experimental Animal Center of The Fourth Military Medical University (Xi’an, China). They were maintained on a 14/10 h light/dark cycle with free access to food and water in the Laboratory Animal Facility of the College of Veterinary Medicine, Northwest A&F University.

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

Collection and Culture of Oocytes and Embryos
Oocytes at the GV stage were obtained from the ovaries of 8–10-week-old females injected with 10 IU of pregnant mare serum gonadotrophin (PMSG) to stimulate the growth of the follicles. Forty-eight hours after PMSG administration the ovaries were placed in Hepes-buffered KSOM medium (H-KSOM) [67] containing 0.1 mM 3-isobutyl-1-methyl-xanthine to inhibit re-summation of meiosis and oocytes were released from the largest follicles by puncturing them with hypodermic needle. The oocytes were completely freed of attached cumulus cells by repeated mouth pipetting.

Mature oocytes arrested in metaphase II were recovered from mice superovulated with intraperitoneal injections of PMSG and human chorionic gonadotrophin (hCG) 48 h apart. Metaphase II oocytes were collected from the oviduct ampullae at 16 h after the hCG injection. Cumulus masses were treated with hyaluronidase (1 mg/ml) to release ova. Metaphase II oocytes and cumulus cells were collected separately.

Zygotes (1-cell embryos) were obtained from females induced to superovulate as described above and mated with males immediately after hCG injection. Zygotes were collected from the oviducts 14 h after hCG injection. Cumulus cells surrounding zygotes were removed by brief exposure to hyaluronidase. Collection of

![Figure 8. Nlrp2 mRNA and protein levels in Nlrp2 knockdown embryos.](image)

The relative abundance of Nlrp2 transcripts in mouse embryos collected at 2 h, 11 h, 20 h, 29 h and 38 h after electroporation. Results have been normalized to the abundance in untreated zygotes and are expressed as the mean ± SEM. Immunoblots of mouse embryos at 4 h (1-cell), 28 h (2-cell) and 52 h (8-cell) after electroporation. doi:10.1371/journal.pone.0030344.g008

![Figure 9. Morphology of Nlrp2 knockdown embryos after being cultured for 3.5 days.](image)

Morphology (left) and DAPI staining (right). The original magnification was ×100. doi:10.1371/journal.pone.0030344.g009
embryos (2-cell, 4-cell, 8-cell, morula and blastocysts) was performed according to previously described protocols [30]. Embryos were cultured in groups of 30–50 in microdrops (100 μl) of KSOMaa medium [68] supplemented with 4 mg/ml bovine serum albumin (KSOMaa-BSA) under liquid paraffin oil in a humidified atmosphere of 5% CO2/95% air at 37°C.

Parthenogenetic Activation of Oocytes

Metaphase II oocytes were subjected to parthenogenetic activation by exposure to activation medium (Calcium-free KSOMaa, 10 mM strontium chloride and 5 μg/ml cytochalasin B) for 6 h. The oocytes were washed in H-KSOM and cultured in KSOMaa-BSA. Pronuclear formation was scored and only activated embryos were used for quantitative RT–PCR, immunoblotting and immunofluorescence.

Collection of Spermatozoa

Epididymides were removed from mature 12-week-old ICR male mice and punctured with hypodermic needles. The tissues were compressed to release spermatozoa into 1.5 ml polypropylene centrifuge tubes; 500 μl of H-KSOM and cultured in KSOMaa-BSA. Pronuclear formation was scored and only activated embryos were used for quantitative RT–PCR, immunoblotting and immunofluorescence.

Cell Culture

RAW264.7 murine macrophage-like cells were maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco). Mouse D3 embryonic stem (D3 ES) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 20% FBS, 2 mM glutamine, 1% nonessential amino acids (Invitrogen, Carlsbad, CA, USA) and 0.1 mM β-mercaptoethanol. 1000 U/ml murine leukemia inhibiting factor (mLIF) was added to the culture medium to maintain ES cell pluripotency. Mouse F9 teratocarcinoma stem cells (F9 ES) were grown in DMEM supplemented with 10% FBS. Mouse D3 ES and F9 ES were plated on gelatinized multiwell tissue culture plates each containing 2 ml of medium. EMT6 mouse mammary carcinoma cells were cultured in Waymouth’s medium (Invitrogen) supplemented with 15% FBS. All cell media were supplemented with 50 U/ml penicillin (Invitrogen) and 50 μg/ml streptomycin (Invitrogen). Cells were cultured at 37°C in a humidified 5% CO2/95% air incubator.

RNA Isolation and Quantitative Real-Time RT–PCR

Total RNA extracts from 4-week-old mouse tissues (ovary, uterus, testis, kidney, lung, heart, liver, brain, stomach, small intestine, muscle and spleen) and different cells (RAW264.7, D3 ES, F9 ES, EMT6, cumulus cells and spermatozoa) were purified with RNasey Mini Kits (Qiagen, Valencia, CA, USA). cDNA synthesis was performed using PrimerScript II 1st Strand cDNA Synthesis Kit (TaKaRa, Otsu, Japan).

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Immunohistochemistry

Ovaries were collected from 3-week-old mice and fixed in 4% paraformaldehyde overnight, dehydrated through graded alcohol solutions and embedded in paraffin wax. Six-micrometer sections were deparaffinized in xylene, rehydrated through graded alcohol solutions and heated in 10 mM sodium citrate buffer (pH 6.0) with microwaves (15 min at medium power) and cooled to room temperature for antigen retrieval. Sections were then incubated with 10% nonimmune goat serum (Maixin-Bio, Kit-7770, Fuzhou, China) to block nonspecific sites followed by incubation with the NLRP2 polyclonal antibody (Sigma-Aldrich, SAB3500325) in blocking solution (1:200 dilution of rabbit anti-NLRP2 antibody; Beyotime, P0102, Jiangsu, China) overnight at 4°C. For enzymatic staining, sections were incubated for 10 min at RT with a biotinylated goat anti-rabbit secondary antibody (Maixin-Bio). Following three 5 min washes in PBS buffer with 0.1% Triton X-100 (PBST), sections were incubated at RT for 10 min with streptavidin-linked alkaline phosphatase (Maixin-Bio) and then washed three times for 5 min each in PBST. 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BICP/NBT) substrate solution (Maixin-Bio) was applied to the sections for 10–30 min and the reaction was stopped by rinsing in PBS. Sections were then mounted with AEC Mounting Solution (Maixin-Bio, AEC-0038). Negative controls consisted of identical reactions with the omission of the primary antibody.

Immunoblotting

To determine protein levels, fifty oocytes or embryos were loaded per lane. Total protein contents were separated by SDS-polyacrylamide gel (8%) and transferred to a PVDF membrane (Millipore, Bedford, MA) using semi-dry transfer. The membrane was blocked for 4 h at RT in Tris-buffered saline (TBS pH 7.4)/

![Figure 11. Overexpression of Nlrp2 in zygotes.](image-url)
0.1% Tween 20 containing 5% dry milk following by an incubation overnight at 4°C with an anti-NLRP2 antibody (Sigma-Aldrich) at a 1:1000 dilution. After several washes in TBS/Tween 20, membranes were incubated for 2 h at RT with a horseradish peroxidase-linked secondary antibody goat anti-rabbit (Pierce, Rockford, IL, USA) at a 1:2000 dilution. The membrane was washed five times in TBS/Tween 20. Finally, the enhanced chemiluminescence (ECL) Advance Western Blotting detection system (Pierce) was used according to the manufacturer’s instructions. Detection of actin, with anti-β actin polyclonal rabbit antibody (1:1000, Santa Cruz Biotechnology Inc., sc-130656, Santa Cruz, CA, USA) and secondary anti-rabbit antibody (1:5000, Pierce) was used as a loading control.

**Immunofluorescence**

Oocytes and embryos were fixed in 4% paraformaldehyde in PBS for 45 min and permeabilized in PBS, 0.2% Triton X-100 for 10 min at RT. After a treatment with 3% BSA in PBS for 1 h, samples were incubated with the primary antibody diluted in PBS containing 1% BSA overnight at 4°C. Rabbit anti-NLRP2 antibodies (Sigma) were diluted 1:100, anti-CDX2 rabbit polyclonal IgG (Santa Cruz, sc-134468) and anti-OCT3/4 mouse monoclonal antibody (Santa Cruz, sc-5279) was diluted 1:50, respectively. After washing in PBS containing 0.3% polyvinylpyrroliodone (PBS/0.3% PVP), samples were incubated 1 h at RT with the secondary antibodies of Cy3-labeled goat anti-rabbit-IgG (Beyotime, A0516) for NLRP2, Alexa Fluor 555-labeled goat anti-
Table 1. Primer sequences for quantitative real-time PCR.

| Genes  | Primer sequences (5’-3’) | Tm (°C) |
|--------|-------------------------|---------|
| Nlrp2  | F: TACTGAATGGTTGAGCTTCTTA 60 |         |
|        | R: CGGTTGATGACCTGGAATCCT 60 |         |
| Nlrp4f | F: CAGGGCTGCTGAGAACTGCA 60 |         |
|        | R: TGAGGTAGTTGAGATGCTGACT 60 |         |
| Nlrp5  | F: CAGGGCTGCTGAGAACTGCA 60 |         |
|        | R: TGAGGTAGTTGAGATGCTGACT 60 |         |
| Nlrp9c | F: AGCAGTGCAAGGACTAAACAA 60 |         |
|        | R: TCAGCATAACAGTGGTGGTTC 60 |         |
| Nlrp14 | F: TTCTCAACGGCAAGGACTAAACAA 60 |       |
|        | R: AGTTTTTGTCCTCACTCAAGG 60 |         |
| β-actin| F: GACAGTGCAAGGACTAAACAA 60 |         |
|        | R: ACTGCCGTTGCAGCATGGAG 60 |         |

*Annealing temperature.
*Forward primer.
*Reverse primer.

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rabbit IgG (Beyotime, A0452) for CDX2 and Alexa Fluor 488-labeled goat anti-mouse IgG (Beyotime, A0428) for OCT3/4. The secondary antibodies were diluted 1:500 with PBS containing 1% BSA. After washing in PBS/0.3% PVP, nuclei were stained with DAPI (Beyotime, C1005) for 5 min. Negative controls were determined by processing samples as described above in the absence of primary antibody. Samples were observed using a Nikon eclipse Ti-S microscope (Nikon) or a Zeiss LSM510 confocal microscope equipped with differential interference contrast optics (Carl Zeiss, Inc., Thornwood, NY).

Immunogold electron microscopic analysis

Ovaries from 10-day-old mice were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) overnight at 4°C and washed thrice in fresh 0.1 M PB and then transferred into 1% osmium tetroxide in 0.1 M PB for 2 h at 4°C. After dehydrating in graded acetone, the ovaries were embedded in epoxy resin (Durcupan ACM, FLUKA, Buchs, Switzerland) and sectioned (70 nm) with a diamond knife mounted in an Ultracut microtome (Leica, Mannheim, Germany). The sections were mounted on nickel grids for immunogold reactions as described [52]. Briefly, the sections were subjected to blocking buffer and then incubated overnight at 4°C in droplets of rabbit anti-NLRP2 antibody (1:500, Sigma-Aldrich). At the end of incubation, the sections were washed thrice to remove excess primary antibody and incubated again in blocking buffer and then incubated overnight at 4°C in droplets of goat anti-rabbit IgG (1:150; Sigma-Aldrich, G7402) for 2 h at RT. After several washings in 0.1 M PB and deionized water, the sections were counterstained with 2% uranyl acetate and lead citrate. Negative controls consisted of samples not incubated with the primary antibody. The sections were observed with a JEM-1230 electron microscope (JEOL Ltd., Tokyo, Japan).

GV-stage oocyte and zygote electroporation with custom siRNA

Electroporation was used to introduce siRNA into mouse oocytes and zygotes as described [30]. GV-stage oocytes or zygotes were collected and washed in H-KSOM, then incubated in prewarmed acid Tyrode’s solution for 12 s to weaken the zona pellucida [29]. They were then washed twice in fresh H-KSOM and kept in the pre-made KSOmaa drops. Afterwards, the siPORT Amine Transfection Agent (Ambion, AM4502, Austin, TX, USA) was diluted in Opti-MEM®I (Invitrogen) medium and incubated for 10 min at RT. The negative control siRNA (Ambion, Silencer® negative control #1, AM4611) or the custom-made Nlrp2 siRNA (mixture of three target-specific 19–25 nt siRNA designed to knock down mouse Nlrp2 expression; Santa Cruz, sc-149011) was diluted into Opti-MEM®I (Invitrogen) medium. The diluted negative control siRNA or the diluted custom-made Nlrp2 siRNA were combined with the diluted siPORT Amine Transfection Agent and incubated for 10 min at RT. The GV-stage oocytes or zygotes were mixed with the transfection complexes. Simultaneously, the cell fusion instrument (BFX Inc., ECM 2001; San Diego, CA) was switched on. The GV-stage oocytes or zygotes were linearly arranged in a flat electrode chamber (BFX Inc.) and electroporated with the negative control siRNA (100 nM) and the Nlrp2 siRNA in different concentrations (20, 40 and 60 nM). The electroporation condition was DC 20 volts/1 ms pulse length/3 pulses/0 repeats. The blank siPORT™ electroporation buffer was used as electroporation control (EP control). The negative control siRNA was used as siRNA control (Control siRNA).

Following electroporation, the GV-stage oocytes were washed thrice and cultured in MEM media supplemented with 3 mg/ml BSA. The maturation rates of oocytes were calculated after 24 h in vitro culture. Some mature oocytes were processed for qRT–PCR and immunoblotting, others were subjected to parthenogenetic activation and the subsequent development was analyzed. After electroporation, zygotes were washed thrice and cultured in KSOmaa-BSA. Some embryos were collected at 2 h (early 1-cell), 11 h (later 1-cell), 20 h (early 2-cell), 29 h (mid 2-cell) and 38 h (late 2-cell) post-electroporation for qRT-PCR. Some were collected at 4 h (1-cell), 28 h (2-cell) and 52 h (8-cell) post-electroporation for immunoblotting.

Construction of NLRP2 expression vector and microinjection

Nlrp2 full-length CDS (3141 bp from 75 to 3215) with Sal I and Xma I sites in the 5’ and 3’ ends was obtained through PCR amplified (sense primer 5’–GGCAATTCCTCTGAGAAGGTTATGATGACAG–3’ and 3’antisense primer 5’–CAAGTTCTAATTCCTCCAG–3’). The PCR product was digested with Sal I and Xma I and inserted to the Sal I/Xma I digested pIRES2-AcGFP1-Nuc (Clontech) eukaryotic expression vector. The recombinant expression plasmid was then sequenced. Zygotes were placed into a micro-culture dish at RT and microinjected with pIRES2-AcGFP1-Nuc-Nlrp2 (pIRES2-Nlrp2). Microinjection was performed using a Narishige microinjector connected to a micromanipulation system (Nikon, Tokyo, Japan). Approximately 5 pl of 0.8 µg/µl solution of expression plasmid was injected into the cytoplasm of per zygote. Zygotes were kept in H-KSOM during injection and then cultured in KSOmaa-BSA. Zygotes injected with pIRES2-AcGFP1-Nuc (pIRES2) were used as control. Embryos were collected for qRT–PCR and immunoblotting assays at different stages.

TUNEL labeling assay

TUNEL (TdT-mediated dUTP nick end labeling) apoptosis assays were carried out with the DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI) according to the manufacturer’s instructions. Apoptosis assays of blastocysts were performed as described [70].
Author Contributions
Conceived and designed the experiments: HP YZ. Performed the experiments: HP BHC JLS YWW PL JLS FSQ ZKG. Analyzed the data: HP BHC YZ. Contributed reagents/materials/analysis tools: HP BHC. Wrote the paper: HP YZ. Editorial assistance: BW Z.

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