Maternally expressed NLRP2 links the subcortical maternal complex (SCMC) to fertility, embryogenesis and epigenetic reprogramming

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Mammalian parental genomes contribute differently to early embryonic development. Before activation of the zygotic genome, the maternal genome provides all transcripts and proteins required for the transition from a highly specialized oocyte to a pluripotent embryo. Depletion of these maternally-encoded transcripts frequently results in failure of preimplantation embryonic development, but their functions in this process are incompletely understood. We found that female mice lacking NLRP2 are subfertile because of early embryonic loss and the production of fewer offspring that have a wide array of developmental phenotypes and abnormal DNA methylation at imprinted loci. By demonstrating that NLRP2 is a member of the subcortical maternal complex (SCMC), an essential cytoplasmic complex in oocytes and preimplantation embryos with poorly understood function, we identified imprinted postzygotic DNA methylation maintenance, likely by directing subcellular localization of proteins involved in this process, such as DNMT1, as a new crucial role of the SCMC for mammalian reproduction.

Infertility and pregnancy loss are common forms of reproductive failure, with primary and secondary infertility affecting 1.9% and 10.5% of women respectively1. Fifteen percent of pregnancies end in a miscarriage and 1% of women who achieve pregnancy will experience recurrent pregnancy loss (RPL), defined as three or more miscarriages2,3. When a pregnancy fails at the embryonic preimplantation stage, before it is clinically recognized, it presents as primary infertility. Knowledge about causes and treatments for infertility and RPL remain unsatisfactory and it is estimated that for 10–15% of couples with infertility and 50% of women with RPL an etiology cannot be identified. One of the earliest required processes for successful reproduction that has been under-studied as a cause of infertility and RPL is the highly dynamic process of transformation from a terminally differentiated oocyte into a pluripotent embryo. Male mammalian germ cells contribute disproportionately to this transformation because prior to zygotic gene activation and initiation of embryonic transcription, the embryo uses maternally-encoded transcripts and proteins for its survival and development4. Thus, the key molecular events that take place during early embryogenesis, including transition from meiosis to mitosis, epigenetic reprogramming and a seamless passage from maternal to zygotic transcription, all depend on maternal proteins5–7. Characterization of mice with inactivation of the genes that encode these proteins has revealed that pregnancies of mutant females often fail to develop beyond preimplantation embryogenesis. A subset of these “maternal effect genes”, namely Nlrp5, Tle6, Ooep, Filia (Khdc3) and Pad6, encode proteins of the subcortical maternal complex (SCMC). This large, multimeric, incompletely understood protein complex, is formed in mature oocytes, persists at the periphery of the outermost cells of the cleavage-stage embryo and is absent from the inner cells and areas of cell-cell contact8–12. Recently, an SCMC containing KHDC3L, NLRP5, OOE6P and TLE6 has also been identified.
in human preimplantation embryos, but the high molecular weights of the human and murine SCMCs indicate that they must contain additional proteins.

In humans, maternal loss of function of some of the genes that encode proteins of the SCMC complex also result in reproductive failure: maternal mutations in PADI6 and TLE6 (PREMBL; OMIM# 616814) cause preimplantation embryonic lethality, while women with KDHC3L mutations experience a recurrent rare specific form of pregnancy loss, recurrent biparental hydatidiform moles (BoHM) (HYDM2; OMIM# 614293). BiHM pregnancies have abnormal hyperplastic vesicular trophoblast with absent fetal development and loss of DNA methylation at differentially methylated regions (DMRs) of all maternally imprinted genes. In addition, women with loss of function mutations in the gene encoding NLRP5 (NLR Family, Pyrin Domain-Containing 5), a member of a subclass of NLR family of proteins that is highly expressed in germ cells and preimplantation embryos, cause relevant phenotypes. Maternal mutations in NLRP7, which is absent from rodent genomes, are a more frequent cause of BiHM (HYDM1; OMIM# 231090) than KDHC3L mutations. Maternal mutations in NLRP2, which is highly homologous to NLRP7 and immediately adjacent to it on chromosome 19, have been described in one family to result in an MLID that presents as recurrent Beckwith-Wiedemann Syndrome due to loss of DNA methylation at the imprinted KdsDMR1 CpG island (CGI), associated with loss of methylation at the DMR of at least one other imprinted locus, PEG1.

The mechanisms by which NLRP2 and NLRP7 impact DNA methylation at imprinted loci are not currently known. Neither is it known whether they are also SCMC members, but if confirmed, it could firmly establish embryonic DNA methylation maintenance as an SCMC function. To examine this in an in vivo model, we generated Nlrp2 null mice on the premise that murine NLRP2 carries out functions that combine those of human NLRP2 and NLRP7, because rodents only have a single Nlrp2 gene while humans have the highly homologous NLRP2 and NLRP7 genes. We observed that loss of maternal NLRP2 results in increased follicular atresia in ovaries of mutant females, subfertility and abnormal cleavage of their embryos when cultured in vitro. In vivo developing pregnancies of Nlrp2-deficient females produce few offspring that exhibit a spectrum of defects including neonatal death, external structural malformations, and stunted and increased growth with evidence of MLID in embryos and neonatally deceased offspring. We also demonstrated that overexpressed NLRP2 interacts with SCMC proteins, TLE6, OOE P, FIIA and NLRP5 and that subcortical localization of TLE6, a key SCMC protein, was grossly altered in oocytes of Nlrp2-null females. Finally, DNMT1, the maintenance DNA methyltransferase, was abnormally localized in oocytes from Nlrp2-null females. These results define a new member of the SCMC complex and identify postzygotic maintenance of DNA methylation at imprinted genes, conferred in part by ensuring appropriate localization of DNMT1, as a function of the SCMC that is critical for preimplantation development and successful pregnancy.

Results

Loss of NLRP2 affects oocyte morphology and follicle maturation dynamics. Reverse transcriptase PCR (RT-PCR) on RNA extracted from brain, liver, kidney, heart, lung, spleen, uterus and ovaries of 3-week-old mice revealed that Nlrp2 expression is restricted to the ovary (Fig. 1A and Supplementary Figure 1). We then generated targeted mice in which Nlrp2 expression was abolished and replaced by transcription of a LacZ element (Supplementary Figure 2) to generate the Nlrp2tm1a allele. Seven Nlrp2tm1a/+ breeding pairs had 23 litters with 32 Nlrp2tm1a/+ and 39 Nlrp2tm1a/+ offspring (Fig. 1B), which was not significantly different from the expected genotype distributions (X2 = 0.0805, P = 0.96). Nlrp2tm1a/+ and Nlrp2tm1a/+ mice were viable, without gross morphological or developmental anomalies, grew normally and did not exhibit a decreased lifespan.

β-Gal staining was only visible in the ovaries of Nlrp2tm1a/+ and Nlrp2tm1a/+ females where it is restricted to oocytes (Fig. 1C). qRT-PCR amplification of expressed Nlrp2 from whole ovaries of 3-week-old mice revealed its complete absence in Nlrp2tm1a/+ mice when normalized to Gapdh (P < 0.0001, Fig. 1D, left panel) or to levels of a germ cell specific gene, Mouse Vasa homolog (Mvh) (P = 0.0021, Fig. 1D, right panel). The latter indicates that its loss of expression was not secondary to a reduction in the number of germ cells, but that it was missing from existing oocytes. Western blotting on oocyte lysates with a new polyclonal antibody to NLRP2 (DKKNPLLPHIFIF), designed to minimize the cross-reactivity with other oocyte-expressed NLRP proteins we observed with commercially available NLRP2 antibodies, showed a specific band at 120 kDa in oocytes from Nlrp2tm1a/+ females but not in oocytes from Nlrp2tm1a/+ females (Fig. 1E). Immunofluorescence staining with the same antibody on paraffin-embedded ovarian sections from Nlrp2tm1a/+ females revealed a fluorescent signal restricted to oocytes that was more intense in the cortical and nucleolar regions (Fig. 1F, upper and middle panels); no signal was seen in oocytes of Nlrp2tm1a/+ females (Fig. 1F, lower panels). We next examined ovarian histology at 3-weeks of age and found more unhealthy, degenerating follicles in ovaries of Nlrp2tm1a/+ females, which were also marked by highly irregular oocyte morphology accompanied by increased nuclear pyknosis of granulosa cells (Fig. 1G), significantly fewer secondary follicles (P = 0.0011; Fig. 1H) and more atretic follicles (P = 0.046, Fig. 1I). No significant differences were noted in primary and antral follicles (Supplementary Figure 3). These data indicate that the ovaries of Nlrp2tm1a/+ have subtle alterations in follicle maturation dynamics. This is to our knowledge the first example of a maternal effect mutation that also produces an ovarian phenotype in homozygous mutant females.
**Nlrp2tm1a/tm1a** mice are subfertile and display a variety of pregnancy outcomes. Eight-week-old wild-type and **Nlrp2tm1a/tm1a** females were bred with >8-week-old **Nlrp2+/+** males to assess fertility and pregnancy outcomes. We recorded outcomes after 30 observed copulatory vaginal plugs in 9 individual **Nlrp2tm1a/tm1a** females and found no offspring born for 13/30 (43%), litters with liveborn offspring for 11/30 (37%), and litters with stillborn pups for 6/30 (20%) observed plugs. Of the 11 litters where liveborn offspring were noted at P0, all pups of 2 litters died within the first few days of life. This was significantly different from outcomes after 15 recorded copulatory vaginal plugs in 10 individual **Nlrp2+/+** mice, of which only 2 (13.3%) did not result in liveborn offspring and 13 (86.7%) were followed by pregnancies that resulted in normal liveborn offspring (χ² = 56.2327,
P < 0.00001, Fig. 2A). Furthermore, the number of pregnancies from which offspring survived to P21 was significantly lower for Nlrp2<sup>−/−</sup> females (N = 9 mice; 30 litters) compared to Nlrp2<sup>+/+</sup> females (N = 10 mice; 15 litters); Outcomes are represented as percentage distribution among all litters. (B) The number of pups alive at weaning was significantly reduced in offspring born to Nlrp2<sup>−/−</sup> females compared to Nlrp2<sup>+/+</sup> females. (C) Craniofacial and skeletal abnormalities noted in Nlrp2<sup>−/+</sup> offspring born to Nlrp2<sup>−/−</sup> females. (D) Severe growth restriction noted in Nlrp2<sup>−/+</sup> offspring born to a Nlrp2<sup>−/−</sup> female compared to Nlrp2<sup>−/+</sup> offspring. Body weight was similar at birth but did not increase over time between P0, P5 and P10. (E) E9.5 Nlrp2<sup>−/−</sup> embryos appear growth restricted compared to Nlrp2<sup>+/+</sup> embryos; representative examples for otherwise normal but small for gestational age (SGA) embryos, morphologically abnormal embryos, and delayed embryos are shown. (F) Number of Nlrp2<sup>−/+</sup> embryos recovered in each pregnancy at E9.5 from Nlrp2<sup>−/+</sup> females does not differ from Nlrp2<sup>−/−</sup> embryos recovered from Nlrp2<sup>−/−</sup> females. (G) The number of SGA Nlrp2<sup>−/−</sup> embryos, which includes small, abnormal and delayed embryos, is significantly higher compared to Nlrp2<sup>+/+</sup> embryos. (H) No difference is noted in the number of abnormal embryos between genotypes. (I) There are significantly more severely delayed Nlrp2<sup>−</sup> embryos compared to Nlrp2<sup>+/+</sup> embryos. All data are shown as mean ± SEM.
**Nlrp2** females or **Nlrp2** males. Considering the combined abnormal follicle maturation and subfertility, we next evaluated if the observed subfertility could be the result of abnormal steroid hormone levels and tested serum levels of anti-Mullerian hormone (AMH), estradiol, progesterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) in **Nlrp2** and **Nlrp2** females, but found no significant differences (Supplementary Figure 4).

To assess if embryonic lethality contributed to the reduced postnatal survival and to examine pregnancies for embryonic structural and growth abnormalities, we performed timed matings of **Nlrp2** or **Nlrp2** females with **Nlrp2** males and dissected pregnant females at E9.5. We noted an array of developmental abnormalities in **Nlrp2** embryos (Fig. 2E), but observed no statistical difference in the number of embryos recovered from either genotype; 9.6 ± 0.3 embryos/litter for three **Nlrp2** females and 7.6 ± 1.3 embryos/litter for five **Nlrp2** females (P = 0.2911, Fig. 2F). However, all 38 (100%) **Nlrp2** embryos were small for gestational age (SGA) of E9.5, compared to only 3 of 29 (10.3%) **Nlrp2** embryos (P < 0.0001, Fig. 2G). Embryos were defined as ‘abnormal’ if they had absence or abnormal morphology of any of the following features: optic vesicle, otic vesicle, branchial arches 1 and 2, and heart, or if they possessed fewer than 21 somites. Embryos were termed as ‘delayed’ if they presented with features of primitive streak embryos (equivalent to less than ~E6.5) or of ~E8 embryos. By these criteria, 17 of 38 (44.7%) of **Nlrp2** embryos were abnormal compared to 3 of 29 (10.3%) of **Nlrp2** embryos (P = 0.09, Fig. 2H) and 10 of 38 (26.3%) of **Nlrp2** embryos were delayed compared to 0 of 29 (0%) of **Nlrp2** embryos (P = 0.0471, Fig. 2I).

In contrast, when **Nlrp2** females were bred with **Nlrp2** females to generate **Nlrp2** offspring, outcomes for 43 pups from 7 litters (6.143 ± 0.8 pups/litter) were no different from those of **Nlrp2** females and **Nlrp2** embryos. Furthermore, there was no increase in adverse outcomes for pregnancies of heterozygous **Nlrp2** females, irrespective of the gender of the **Nlrp2** parent. This indicates that, in contrast to the proposed human tissue-specific **NLRP2** imprinting	extsuperscript{2,3}, murine **Nlrp2** is not imprinted. Taken together these data show that **Nlrp2** is a maternal effect mutation.

**Maternal loss of NLRP2 results in decreased fertilization rates, abnormal early cleavage and a striking inability to form blastocysts in vitro.** To further examine the role of maternal NLRP2 in early embryo development and characterize the origins of the observed delay in embryogenesis of **Nlrp2** embryos at E9.5, we recovered oocytes and zygotes from superovulated dams after mating with proven males for in vitro culture with imaging of developing embryos from the 1-cell stage to blastocyst hatching on day 5 using an EmbryoScope time-lapse imaging system. Embryos were presented in Video S1 (showing two **Nlrp2** embryos). By these criteria, 17 of 38 (44.7%) of **Nlrp2** embryos were abnormal compared to 3 of 29 (10.3%) of **Nlrp2** embryos (P = 0.09, Fig. 2H) and 10 of 38 (26.3%) of **Nlrp2** embryos were delayed compared to 0 of 29 (0%) of **Nlrp2** embryos (P = 0.0471, Fig. 2I).

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**NLRP2 interacts with SCMC proteins TLE6, OOEP, FILIA and NLRP5.** The cleavage abnormalities observed in **Nlrp2** embryos are similar to those seen in embryos of females with mutations in genes that encode SCMC proteins, which indicated that NLRP2 may be a member of the SCMC. We therefore tested co-immunoprecipitation of overexpressed proteins in HEK293T cells if NLRP2 interacts with SCMC proteins and encode SCMC proteins, which indicated that NLRP2 may be a member of the SCMC. We therefore tested by

![NLRP2 interacts with SCMC proteins TLE6, OOEP, FILIA and NLRP5.](https://example.com/nlrp2_interacts_with_scmc_proteins.png)

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Given that SCMC proteins have a characteristic localization in the subcortical region of the oocyte and outermost cells of the preimplantation embryo, we examined expression and subcellular localization of NLRP2 and found that it binds to TLE6, OOEP, FILIA and NLRP5 (Fig. 4A and Supplementary Figure 5).

To investigate this but minimize effects on oocytes and embryo cleavage of in vitro culture, we dissected the oviducts of superfused females and performed immunofluorescence staining overnight at 4°C on paraffin-embedded oocytes with an antibody to TLE6, a representative marker of the SCMC. We found that, compared to oocytes from **Nlrp2** females in which TLE6, consistent with published data, was highly concentrated in a narrow subcortical rim, oocytes from **Nlrp2** females showed much more intense and diffuse TLE6 staining (Fig. 4C).

To address if the discrepant staining pattern observed in SCMC proteins depends on processing procedures	extsuperscript{2,3}, we conducted this staining by a recommended alternate condition that included 1 hour staining at room temperature. Contrary to previous reports we did not observe any difference in TLE6 staining in control oocytes from **Nlrp2** females with different processing conditions. However, staining for 1 hour at room temperature in oocytes from **Nlrp2** females showed much weaker TLE6 staining (Supplementary Figure 6). This observation contrasts the findings when the staining is carried out overnight at 4°C and are more consistent with other reports where knockout of one of the SCMC components results in loss of characteristic expression of other
Figure 3. Maternal loss of NLRP2 results in reduced fertilization rates, abnormal early cleavage and inability to form blastocysts in in vitro developing embryos. (A) Representative still frames at 36, 60, 96 hours and at the end of imaging (~120 hours) from Nlrp2M+/Z+ and Nlrp2M−/Z+ embryos. Most Nlrp2M−/Z+ embryos arrest at 2-cell stage or degenerate, or rarely, have delayed compaction (white arrowhead) almost 60 hours after it has initiated in Nlrp2M+/Z+ embryos. (B) No difference in the number of ovulated oocytes is noted between genotypes, but (C) a significant decrease in the fertilization rates and (D) blastocyst formation is noted in Nlrp2M−/Z+ embryos compared to Nlrp2M+/Z+ embryos. All data are shown as mean ± SEM.
Figure 4. NLRP2 interacts with SCMC components TLE6, OOEP, FILIA and NLRP5. (A) NLRP2 was overexpressed with myc-tagged OOEP, TLE6, NLRP5 and FILIA in HEK293T cells for 48 hours, immunoprecipitated with anti-NLRP2 or IgG as negative control and immunoblotted with anti-myc. Top panel shows specificity of anti-NLRP2 IP and bottom panel shows that NLRP2 binds to OOEP, TLE6, FILIA and NLRP5. Uncropped, full length western blots have been provided in Supplementary Figure 5. (B) Whole-mount immunofluorescence co-staining with anti-NLRP2 (green) and DAPI (blue) for nuclear staining on Nlrp2^+/+ oocytes and embryos at 2-, 4-, 16-cell and morula stages revealed a predominantly SCMC-like localization for NLRP2. Scale bars represent 200 μm. (C) Co-staining with anti-TLE6 (green) and DAPI (blue) of paraffin-embedded oocyte sections reveals a typical cortical stain of TLE6 in oocytes of Nlrp2^+/+ dams but a more intense and diffuse stain in oocytes of Nlrp2^{tm1a/tm1a} dams. Scale bars represent 10 μm.
Abnormal DNA methylation at imprinted loci in stillborn offspring and embryos of Nlrp2^tm1a^tm1a females. Considering that in humans, maternal effect mutations in NLRP2 and other members of the NLRP family (NLRP7 and NLRP5), as well as KHD3CL affect imprinting DNA methylation in trophoblast or offspring, we next investigated if the embryological cleavage defects, embryonic growth restriction, abnormal development and stillbirth of offspring from Nlrp2^tm1a^tm1a females could also be a consequence of abnormal imprinting DNA methylation. We analyzed DNA methylation at DMRs of maternally imprinted genes Impact, Nesp, Zac1, and Kcnq1ot1 and maternally expressed Cdkn1c on bisulfite-converted genomic DNA extracted from stillborn pups. In addition to the above listed genes, we also analyzed Srpnp, Pegr3, Iigf2r and paternally imprinted genes H19 and Gilt2 on bisulfite-converted genomic DNA extracted from E9.5 embryos. We found a non-significant trend for a mixed pattern of gain and loss of methylation at Nesp and Kcnq1ot1 and for loss of methylation at Cdkn1c in stillborn pups. However, we observed a significant gain of methylation at Zac1 (P = 0.0424) and Impact (P = 0.0091) in stillborn Nlrp2^M/^Z+ embryos (Fig. 5A and B), together with a more selective gain of methylation at Zac1 in Nlrp2^M/^Z− embryos at E9.5 (P = 0.029, Fig. 5C and D). We did not observe an increase in methylation at Mest, however Nlrp2^M/^Z− embryos exhibit significantly greater variability in levels of methylation compared to Nlrp2^M/^Z− control embryos (F statistic for variance P = 0.027). Supplementary Table 2 contains specific methylation levels for all the analyzed pups and embryos. To assess if the alterations in DNA methylation were associated with abnormal expression of tested imprinted genes, we analyzed expression of maternally imprinted genes Impact, Mest, Grb10, Srpnp, Peg3, Cdkn1c and Igf2r and of the paternally imprinted gene H19. We found that the gain in methylation in Zac1 was accompanied by decreased expression of Zac1 (P = 0.0459) and also noted that Mest, which had more variable methylation, had lower expression (P = 0.048) (Fig. 5E). The observation that maternal deficiency of NLRP2 results in mixed losses and gains of methylation indicates a possible stochastic feature to its function. Intriguingly, we also found that while methylation of the paternally imprinted genes H19 and Gilt2 were not significantly different, they were more variable in E9.5 Nlrp2^M/^Z− embryos compared to Nlrp2^M/^Z− control embryos (Fig. 5C), indicating that altered embryonic DNA methylation subsequent to absence of maternally contributed NLRP2 is not restricted to maternally imprinted genes.

To investigate the mechanisms by which lack of a maternally provided protein could impact methylation at both maternally and paternally imprinted genes, we examined the subcellular localization of DNA methyltransferase 1 (DNMT1) in oocytes and embryos from Nlrp2^+/+ and Nlrp2^tm1a^tm1a females. We found that in oocytes and Nlrp2^M/Z+ preimplantation embryos of Nlrp2^+/+ females, expression and localization of DNMT1 was similar to that of NLRP2 and other SCMC proteins, with a striking cortical concentration of DNMT1 along with a weaker, more diffuse cytoplasmic signal (Fig. 6A). Because preimplantation development of Nlrp2^M/Z−/− embryos is severely constrained, we investigated the expression and localization of DNMT1 in ovulated oocytes of Nlrp2^tm1a^tm1a females by immunofluorescence staining on paraffin embedded postovulatory oocytes. We found that like TLE6, DNMT1 had a stronger and more diffuse localization in fertilized oocytes of Nlrp2^tm1a^tm1a females compared to those of Nlrp2^+/+ females, potentially suggesting that the disruption of the SCMC affects proper localization of DNMT1. Additionally, a denser concentration of DNMT1 was noted in the nucleoli of oocytes from Nlrp2^tm1a^tm1a females (Fig. 6A). We then assessed expression and localization of DNMT3A and DNMT3B in control and targeted oocytes as previously described. As expected from published reports, DNMT3A is associated with metaphase chromosomes in oocytes of Nlrp2^+/+ females and no difference is noted in oocytes of Nlrp2^tm1a^tm1a females (Fig. 6C). DNMT3B is not expressed in oocytes and therefore no expression is noted in either control oocytes or those derived from Nlrp2^tm1a^tm1a females (Fig. 6D).

Discussion
At the onset of this study, little was known about the function of NLRP2, except that it is abundant in early embryos, that in one family its maternal loss in humans causes an imprinting disorder in offspring and that it is highly homologous to NLRP7, maternal mutations of which cause recurrent and abnormal pregnancies with multilocus imprinting defects. Considering that NLRP7 is absent from the mouse genome, we speculated that the product of the murine Nlrp2 gene might carry out functions that combine those of human NLRP2 and NLRP7.

Furthermore, that mice lacking Nlrp2 would exhibit an inability to establish healthy pregnancies. We indeed found that Nlrp2^tm1a^tm1a male and female mice were indistinguishable from their wild type and heterozygous littermates for growth, mating behavior and survival. While Nlrp2-deficient males had normal fertility, females had a wide range of reproductive outcomes. There were fewer and smaller litters, increased neonatal death and a mixture of offspring with abnormal growth and multiple developmental defects. Despite the lower numbers of offspring observed after full-term in vivo pregnancies, we found that although most embryos were small or abnormally developed at E9.5, the number of embryos was not different from Nlrp2^+/+ mice, suggesting that resorption occurred later. In contrast, Nlrp2^M/Z−/− embryos that were flushed from the oviduct after mating of superovulated females and then cultured in vitro became heavily fragmented and did not form blastocysts. This surprising finding suggests that in mice the embryonic phenotype is exacerbated by hormonal ovulation induction, embryo culture or their combination.

These studies are reminiscent of the findings from in vitro siRNA knockdown of Nlrp2 which resulted in mostly fragmented and degenerated embryos that were unable to reach the blastocyst stage. In humans, the primary presentation of NLRP2 maternal effect mutations, is a Beckwith-Wiedemann phenotype in offspring, whereas mutations in NLRP7 cause recurrent biparental hydatidiform moles, a placental developmental phenotype. To address if Nlrp2 maternal effect mutations are also associated with placental abnormalities, it will be important to conduct detailed investigations of placental morphology and cell-type specific imprinting disturbances in sorted placental cell populations from pregnancies of Nlrp2^tm1a^tm1a females.
The inability of Nlrp2−/Z+ embryos to cleave normally in vitro is very similar to what is found in mice with mutations in genes encoding members of the SCMC, thus we were not surprised to find that NLRP2 binds to SCMC proteins, localizes in a pattern that overlaps significantly with the subcortical localization of the SCMC in oocytes and preimplantation embryos and that its loss alters the distribution of TLE6, a core SCMC protein. Intriguingly, in contrast to females with inactivation of Nlrp5, Tle6 or Ooep, who never achieve a successful pregnancy, 37% of embryos and offspring of Nlrp2−/Z+ females were able to develop post-implantation and a subset of these were apparently normal at birth. This suggests that although NLRP2 is a member of the SCMC, either
Figure 6. DNMT1 reveals a SCMC-like cortical localization with aberrant localization in Nlrp2⁴⁺/⁺ derived oocytes. (A) Whole mount immunofluorescence co-staining with anti-DNMT1 and DAPI on the same Nlrp2²⁺/⁻ embryos used to stain NLRP2 reveals a SCMC-like cortical localization of DNMT1 with a diffuse nuclear stain in later stage embryos. Scale bar represented is 200 μm. (B) DNMT1 staining in paraffin embedded oocyte sections reveals a less cortical, more cytoplasmic intense stain with nucleolar focus in oocytes from Nlrp2²⁻/⁻ dams compared to oocytes from Nlrp2²⁺/⁺ dams. (C) Whole mount immunofluorescence for DNMT3A in unfertilized control oocytes reveals a characteristic metaphase associated localization and no difference is noted in oocytes derived from Nlrp2²⁻/⁻ dams. (D) Whole mount immunofluorescence in unfertilized oocytes reveals that as expected, DNMT3B is not yet expressed in unfertilized control oocytes and no expression is noted in oocytes derived from Nlrp2²⁻/⁻ dams.
the integrity of the SCMC is not fully dependent on NLRP2, or in some cases development can proceed even in the absence of an intact SCMC. Further research will be needed to differentiate between these two possibilities.

The variable phenotypes of Nlrp2/+ pregnancies and offspring indicate that the consequences of maternal loss of Nlrp2 on reproductive outcomes are stochastic, which suggests that an epigenetic mechanism is involved. We therefore examined if murine Nlrp2, similar to human NLRP2 and NLRP7, causes loss of DNA methylation at DMRs of imprinted genes. We found that, unlike the complete loss of DNA methylation at maternally imprinted genes noted in the pregnancies of women with NLRP7 loss of function mutations, Nlrp2M−/− offspring and embryos exhibited a wide range of DNA methylation gains and losses. The increase in DNA methylation and reduced mRNA from the Zac1 locus in Nlrp2M−/− offspring is intriguing, because the developmental abnormalities in the paternal knockout of Zac1 are strikingly similar to those in Nlrp2M−/− offspring and we speculate that reduced expression of Zac1 causes some of the phenotypes we observed, but aberrant gains and losses in DNA methylation causing altered expression at other imprinted genes may also contribute. We next examined if the altered DNA methylation at imprinted loci could be due to erroneous expression of DNA methyltransferases that are active during the 1 to 8 cell stage. The somatic variant of DNMT1 is believed to contribute, at least in part, to maintenance of DNA methylation at imprinted loci during preimplantation embryogenesis, and especially prior to nuclear shuttling of the oocyte provided DNMT1 is active. Following fertilization, DNMT3A remains nuclear and its staining intensity is lower by blastocyst stage. In contrast, DNMT3B is not expressed in oocytes or in preimplantation embryos until the 4-cell stage. Our whole mount immunofluorescence in oocytes confirmed DNMT3A’s association with metaphase chromosomes and absence of DNMT3B in oocytes from wild-type and Nlrp2M−/− embryos. Due to the difficulties in culturing embryos from Nlrp2M−/− dams beyond the two-cell stage, we restricted our current analysis to oocytes, but plan to examine in future experiments if expression in developing embryos from Nlrp2M−/− dams is abnormal. Understanding these differences will provide greater insight into the specific mechanisms by which SCMC proteins affect postzygotic imprinted DNA methylation. Interestingly, embryos obtained from oocytes that lack maternal KDM1A, another epigenetic factor that contributes to imprinting, also exhibit hypermethylation at Zac1 and Impact11.

One of our objectives was to understand if loss of maternal NLRP2 compromised fertility and impacted embryonic development. We found a wide range of phenotypes of which the mid-gestation embryos and pups that developed to term probably represent the milder spectrum and associated imprinting defects caused by maternal loss of NLRP2. It will be important to assess in the future, imprinting in oocytes and preimplantation embryos. These studies can clarify if murine NLRP2 functions more like human NLRP7 which appears to be necessary for imprint acquisition18,32,33, or like human NLRP5 which appears to function during imprint maintenance. Because murine NLRP2 likely carries out the functions of human NLRP2 and NLRP7, establishing whether murine NLRP2 functions in imprint acquisition or maintenance may also shed light on the function of human NLRP7.

An important new observation is that mouse NLRP2 interacts with proteins of the SCMC. This is complemented with our unpublished data that human NLRP7 and NLRP2 interact with SCMC components KHDC3L, TLE6 and OOEP. Furthermore, the localization of murine NLRP2 also resembles that of NLRP7’s cortical localization with decreasing intensity towards regions of cell to cell contact. Collectively, this indicates that NLRP2 and NLRP7 are themselves SCMC members. Thus, our data on altered DNA methylation, together with the abnormal DNA methylation noted in pregnancies of women with mutations in NLRP7 and KHDC3L, strongly supports that one of the functions of the SCMC is postzygotic maintenance of DNA methylation. This is surprising given the subcortical cytoplasmic localization of SCMC proteins, since DNA methylation is a nuclear process. Considering that certain SCMC mutants have decreased maternal stores of RNA and protein, one could speculate that in addition to the observed changes in DNMT1, stores of proteins such as PGCG, KAP1 and ZFP57, which all function to maintain DNA methylation in the preimplantation embryo could be mislocalized, depleted or perhaps not appropriately degraded when the embryonic genome becomes activated. While the striking cortical localization of DNMT1 suggests that it could be bound to the SCMC, it could also be that reserves of critical maternal proteins are localized at the cortical rim of the developing embryo, such that destabilization of the SCMC indirectly impacts nearby DNMT1, but other processes may also contribute. For example, a proposed major function of the SCMC is to ensure faithful maintenance of ploidy and symmetric cell division. Thus, abnormal cleavage might affect the cell cycle of individual cells within a developing embryo, further impacting appropriate localization of DNA methyltransferases. A final scenario may simply be that SCMC-mutant oocytes and the embryos derived from them lack the appropriate stoichiometry of maternal proteins such that abnormal DNA methylation is a bystander effect to a generalized state of crisis in the embryo. Further studies will be needed to differentiate between these possible mechanisms.

In conclusion, we show that in mice, maternal loss of NLRP2 results in significantly compromised fertility and defective embryogenesis with variably penetrant multi-locus imprinting disturbances. This supports the observation that embryos deficient for at least one of the maternally contributed SCMC proteins can have aberrant DNA methylation, either directly or as a bystander to the generalized developmental disruption of the embryos. This information also suggests that abnormal DNA methylation should be investigated in pregnancies of other SCMC mutants. The observed reproductive outcomes and impact on DNA methylation also indicate that there is both functional overlap and divergence between the murine Nlrp2 and human NLRP2 and NLRP7 genes. The findings
from this study further imply that women with unexplained primary infertility due to preclinical pregnancy loss or with early arrest of all embryonic development during IVF and pervasive failure of development following IVF may harbor mutations in this and other genes that encode SCMC proteins or in genes that encode other oocyte-expressed proteins that are important for reprogramming of DNA methylation. While the mutational frequency of SCMC genes in unexplained infertility and women with enduring failure of development following IVF remains to be determined, it is an important cause to recognize, as it would not be treatable by in vitro fertilization, unless donor oocytes are used. Finally, this work combined with prior studies and clinical observations define maternal effect gene mutations as a new category of genetic defects that decrease reproductive fitness.

Materials and Methods

The study protocol (AN-2035) was approved by the Institutional Animal Care and Use Committee (IACUC) at Baylor College of Medicine (BCM). All experiments were conducted according to institutional and governmental regulations concerning the ethical use of animals. All animal facilities are approved by the Association for Assessment and Accreditation for Laboratory Animal Care International (AAALAC).

Assessment of fertility. 7–8 week old female \(Nlrp2^{+/+}\) and \(Nlrp2^{mla/mla}\) mice were housed with >8 week old \(Nlrp2^{+/+}\) male mice between 1600–1800 hours and checked for the presence of vaginal plug the following morning. If a vaginal plug was observed, the male was removed from the cage and females monitored closely beginning at E18.5, for parturition. At birth, the gestational length, number of offspring born and gender distributions were noted. In instances where live, morphologically abnormal offspring were found, body weights were recorded and photographs were taken. In instances where offspring were found deceased, photographs were taken and tissues preserved for subsequent analysis. If the female did not deliver beyond 25 days post coitus, the pregnancy outcome was recorded as “no outcome”.

Western blotting. Three commercially available NLRP2 antibodies were tested in Western Blotting and Immunofluorescence (IF) / Immunohistochemistry (IHC) applications. We tested Anti-NLRP2 antibody produced in rabbit (Sigma-Aldrich, St. Louis, MO; Cat #SAB1411064), Anti-NALP2 antibody produced in rabbit (Sigma-Aldrich, St. Louis, MO; Cat #SAB3500325) and NLRP2 MaxPab mouse polyclonal antibody (B01) (Abnova, Taipei; Cat # H00055655-B01) on either whole oocyte protein lysates for western blotting or paraffin embedded ovary sections for IHC.

Due to the non-specificity of commercially available mouse reactive NLRP2 antibodies, we generated a goat polyclonal antibody against the C terminal DLKNPLPHFIF sequence (Bethyl Laboratories, Inc.). Western blotting on oocyte lysates were performed as follows. 30 denuded oocytes were denatured at 100°C for 10 minutes in 4X Bolt LDS Sample Buffer (Life Technologies, Cat #B0007) and 10X Bolt Sample Reducing Agent (Life Technologies, Cat #B0004). Oocyte lysates were run on Bolt 4–12% Bis-Tris Plus Gels (Life Technologies, Cat #NW04120BOX) and transferred to a nitrocellulose membrane. Membranes were blocked in 5% blottng grade blocker (Bio-Rad, Cat # 1706404) in PBS, 0.1% Tween-20. Primary antibodies were diluted in the blocking solution at 1:250. HRP conjugated Donkey anti goat IgG HRP (Santa Cruz Biotechnology, Cat # sc-2020) secondary antibody was used. Substrates were detected using the SuperSignal West Femto chemiluminescent substrates (Thermo Fisher Scientific, Cat #34077, 34094). All western blots were carried out on a minimum of 3 per genotype and repeated thrice.

Immunofluorescence. Immunofluorescence was carried out on 4μM sections from paraffin embedded oocytes and ampulla collected from superovulated females. The tissues were processed as listed under histological studies. Sections were de-paraffinized and dehydrated following which a Citrate based antigen retrieval solution (pH6) was used to recover the epitopes. Sections were blocked in 5% normal donkey serum in 1X PBS, 0.1% Tween-20 and the same blocking solution was used to dilute primary and secondary antibodies. Primary antibodies were incubated in humidified conditions overnight at 4°C. The following antibodies were used at the indicated dilutions: Anti-NLRP2 (Bethyl, custom generated polyclonal, 1:50), anti-TLE6 (Santa Cruz Biotechnology, Cat #sc-162320, 1:50). Secondary antibodies used were Alexa Fluor 594 Donkey Anti-Rabbit IgG (Thermo Fischer Scientific, Cat # A-21207) and Alexa Fluor 488 Rabbit anti-Goat IgG (Thermo Fischer Scientific, Cat #A-21222, 1:50). To assess if processing conditions alter the observed TLE6 staining pattern, the primary antibody incubation was also carried out at room temperature for 1 hour. The remainder of the protocol was unchanged. NucBlue® Fixed Cell ReadyProbes® Reagent (Thermo Fischer Scientific, Cat #R37606) was used to stain nuclei. ProLong® Gold Antifade Mountant (Thermo Fischer Scientific, Cat # P36930) was used to mount the slides. Slides were imaged using a Zeiss LSM 880 with Airyscan Confocal Microscope at the Neurological Research Institute Microscopy core. Whole mount immunofluorescence was performed as follows: P28 mice were superovulated as described earlier and mated with \(Nlrp2^{+/+}\) males. The following morning, E0.5 zygotes were collected form the ampulla, denuded and cultured as described under embryoscope studies. At 24 hour intervals, zona containing embryos were rinsed in M2 media and fixed in 4% PFA for 20 minutes at room temperature. Embryos were then permeabilized with 1% Triton X-100 in 1X PBS prior to being blocked in 1% NDS (normal donkey serum) in the permeabilization solution. Antibodies were diluted in the blocking solution as follows: Anti-NLRP2 (primary 1:250 overnight at 4°C, secondary Alexa Fluor 488 1:1000 1 hour at room temperature), Anti-DNMT1 (Santa Cruz Biotechnology, Cat #sc-20701 primary 1:250 overnight at 4°C, secondary Alexa Fluor 594 1:1000 1 hour at room temperature). For staining with anti-DNMT3A (Novus Biologicals, Cat #NB120-13888SS) and anti-DNMT3B (Novus Biologicals, Cat # NB100-56514SS), the following conditions were used: Superovulated oocytes were fixed for 30 minutes in 3.7% paraformaldehyde on ice. Following washes in 1X PBS, oocytes were treated with a pre-treatment buffer (2% Triton X-100 in 1X PBS) for 30 minutes. Oocytes were then transferred to primary antibody solutions (1:500 diluted in 0.1% Triton X-100 in 1X PBS) overnight at 4°C. Following washes
with 1X PBS, oocytes were transferred to secondary antibody solutions (Alexa Fluor 488 1:2000 diluted in 0.1% triton X-100) for 30 minutes at room temperature. Oocytes and embryos were mounted on glass slides and imaged as described above. All laser intensities and saturations were maintained consistently during imaging. Raw images were deconvoluted using AutoQuant X3 and further processed using Imaris version 8.2.

**Embryo culture.** Superovulated oocytes were rinsed in Quinns Advantage Medium with HEPES (Origio, Cat #12-604 F/12) with 10% Fetal Bovine Serum and 1X antibiotics at 37 °C with 5% CO2. The following plasmids were transfected into HEK293T cells using the PolyJet™ In Vitro DNA Transfection Reagent (SignaGen Laboratories, Cat #SL100688). N’FLAG-NLRP2 (Genecopoeia, Cat #EX-Mm25920-M11), C’MyC-FILIA (Genecopoeia, Cat # EX-Mm08903-M09), C’MyC-DDK-TLE6 (OriGene, Cat #MR208164), C’MyC-DDK-OOE (OriGene, Cat #MR201316) and C’MyC-NLRP5 (Genecopoeia, Cat # EX-Mm21358-M09). Because the co-transfection efficiency of NLRP2 and FILIA/NLRP5 was low, we used Lipofectamine 2000 (Thermo Fisher Scientific, Cat # 11668027). The remainder of the steps were processed the same, regardless of transfection agent. Plasmids were overexpressed in HEK293T cells for 48 hours, following which protein was isolated using a NP-40 based lysis buffer. Protein A Dynabeads (Thermo Fisher Scientific, Cat # 10002D) were conjugated with 5 μg of anti-NLRP2 antibody and used to immunoprecipitate 300 μg of protein. IgG-conjugated dynabeads were used for pre-clearing the lysates and for serving as a negative control. 10% of protein was reserved as input. Immunoprecipitated products and inputs were analyzed by western blotting as described in an earlier section. Interacting proteins were detected using an anti-Myc antibody (Novus Biologicals, Cat #NB600-302).

**NLRP2 interactor studies.** HEK293T cells were cultured in Dulbecco’s Modified Eagle Medium (Lonza Cat #12-604 F/12) with 10% Fetal Bovine Serum and 1X antibiotics at 37 °C with 5% CO2. The following plasmids were transfected into HEK293T cells using the PolyJet™ In Vitro DNA Transfection Reagent (SignaGen Laboratories, Cat #SL100688). N’FLAG-NLRP2 (Genecopoeia, Cat #EX-Mm25920-M11), C’MyC-FILIA (Genecopoeia, Cat # EX-Mm08903-M09), C’MyC-DDK-TLE6 (OriGene, Cat #MR208164), C’MyC-DDK-OOE (OriGene, Cat #MR201316) and C’MyC-NLRP5 (Genecopoeia, Cat # EX-Mm21358-M09). Because the co-transfection efficiency of NLRP2 and FILIA/NLRP5 was low, we used Lipofectamine 2000 (Thermo Fisher Scientific, Cat # 11668027). The remainder of the steps were processed the same, regardless of transfection agent. Plasmids were overexpressed in HEK293T cells for 48 hours, following which protein was isolated using a NP-40 based lysis buffer. Protein A Dynabeads (Thermo Fisher Scientific, Cat # 10002D) were conjugated with 5 μg of anti-NLRP2 antibody and used to immunoprecipitate 300 μg of protein. IgG-conjugated dynabeads were used for pre-clearing the lysates and for serving as a negative control. 10% of protein was reserved as input. Immunoprecipitated products and inputs were analyzed by western blotting as described in an earlier section. Interacting proteins were detected using an anti-Myc antibody (Novus Biologicals, Cat #NB600-302).

**Bisulfite sequencing.** Genomic DNA was isolated from the facial regions of the deceased pups born to Nlrp2""""+/-"""" females. All pups had a genotype of Nlrp2""""+/-"""". Control pups also had an Nlrp2""""+/-"""" or Nlrp2""""+/-"""" genotype but were born to Nlrp2""""+/-"""" females. 3–4 pups from 3 Nlrp2""""+/-"""" females were used as controls. Six to nine pups from 3 Nlrp2""""+/-"""" females were used for analysis. E9.5 embryos were used as a whole for DNA isolation. DNA was isolated using the Gentra Puregene Blood Kit (Qiagen, Cat # 158445) and 200 ng–1 μg of DNA was bisulfite converted using the EZ DNA Methylation-Direct Kit (Zymo Research, Cat # D5020). Six embryos from 2 Nlrp2""""+/-"""" females and 8 embryos from 2 Nlrp2""""+/-"""" females were used for analysis. Depending on the developmental competence of the E9.5 embryos, we recovered 50 ng to several micrograms on DNA. In instances where the amount of DNA was less than 200 ng, bisulfite conversion was carried out on as much DNA as was available. Primers used to amplify imprinted loci were provided in Supplementary Table 3. PCR products were cloned into the PCR4 TOPO TA cloning vector (Life Technologies, Cat # K4575-02). Sixteen to thirty-two clones were selected following blue white screening for sequencing using the M13 reverse primer (GeneWiz, New Jersey). CpG viewer provided by the Leeds Institute of Molecular Medicine was used to generate scaled lollipop diagrams (Carr IM, Valleye EMA, Cordery SF, Markham AF & Bonthron DT (2007). Sequence analysis and editing for bisulfite genomic sequencing projects. Nucleic Acids Res., 35:e79). An in-house script was written in Python to quantify percentage of methylated versus unmethylated CGs in the sequences. CpG viewer was used to align chromatograms with the bisulfite converted reference sequence. CpG Viewer was then used to export files as lollipop diagrams (as shown in Fig. 5 and Supplementary Figure 7) or text files. The python script was written to scan the text files for “CG” dinucleotides and quantify the number of CG versus TG dinucleotides per sample analyzed which finally provides methylation percentage. Several lollipop diagrams per gene were manually compared to the methylation percentage generated by the script to ensure accuracy of the generated methylation levels.

**Statistical tests.** All plotted data were represented as mean ± SEM. An unpaired student’s t-test was used to calculate significance and P < 0.05 was used as the significance cut-off for normally distributed data. For count-based data, a nonparametric Mann Whitney U test was used to estimate significance. Chi square analysis was carried out using a web based calculator (http://www.socscistatistics.com/tests/chisquare2/Default2.aspx) and a significance of P < 0.05 was used as a cutoff. All data were generated using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

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Author Contributions
S.M. designed and performed most of the experiments and data analysis, prepared figures and wrote all the drafts of the paper. V.S. performed follicular count assays and assisted with standardization of immunofluorescence staining conditions. B.U. assisted with processing of confocal microscopy images. I.L. was involved in electroporation of the Nlrp2 targeting construct into ES cells and advised on superovulation experiments. K.K. was involved with setting up embryoscopy experiments and culturing embryos for whole mount immunofluorescence experiments. I.B.V. was responsible for project design, oversight of all experiments and interpretation, final manuscript and figure preparation.

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Erratum: Maternally expressed NLRP2 links the subcortical maternal complex (SCMC) to fertility, embryogenesis and epigenetic reprogramming

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These errors have now been corrected in the PDF and HTML versions of this Article.

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