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Expression patterns of *Nlrp9a*, *Nlrp9b* and *Nlrp9c* during mouse development

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Abstract: The *Nlrp* gene family contains 20 members in the mouse. Recent studies have demonstrated that these genes play key roles in reproduction. In this study, we investigated the expression patterns of *Nlrp9a*, *Nlrp9b* and *Nlrp9c* in the mouse. In 2-week-old mouse tissues, *Nlrp9a*, *Nlrp9b* and *Nlrp9c* were all strikingly expressed in the ovary, while the transcripts of *Nlrp9b* and *Nlrp9c* were also detected in other tissues. The transcripts of *Nlrp9a*, *Nlrp9b* and *Nlrp9c* were restricted to the oocytes and declined with oocyte aging within the ovary. Furthermore, *Nlrp9a*, *Nlrp9b* and *Nlrp9c* transcripts presented evidence for the exclusive maternal origin, which were presented in oocytes and zygotes, immediately downregulated and not detected after the 2-cell stage during preimplantation development. In addition, *Nlrp9a* and *Nlrp9c* transcripts were not detected in other cells except for oocytes. Nevertheless, *Nlrp9b* expression was detected in oocytes, as well as in D3 ES and F9 ES. These results indicate that *Nlrp9a*, *Nlrp9b* and *Nlrp9c* display specific or preferential oocyte expression patterns and may play critical role in oogenesis and/or preimplantation embryo development in the mouse.

Key words: Expression analysis; *Nlrp9a*; *Nlrp9b*; *Nlrp9c*; mouse

Abbreviations: NLRP: Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing Proteins. Mater: Maternal antigen that embryos require. GV: Germinal vesicle. PMSG: Pregnant mare serum gonadotropin. hCG: human chorionic gonadotrophin. KSOM: Protein-free potassium (K) Simplex Optimized Medium. FBS: Fetal bovine serum. ES: Embryonic stem cell. RT–PCR: Reverse transcription polymerase chain reaction. qRT–PCR: quantitative real-time polymerase chain reaction. ISH: In Situ Hybridization.

Introduction

Preimplantation embryo development is largely relied on maternal transcripts and proteins synthesized and stored during oogenesis (Latham 1999; Latham & Schultz 2001). The reliance on maternal transcripts is on account of mature oocytes and zygotes are transcriptionally quiescent but translationally active. In the mouse, the maternal-to-zygotic transition occurs during the late one-cell/early two-cell stages (Ko et al. 2000; Schultz 2002; Wang et al. 2004), while a major burst of zygotic genome activation occurs at the late two-cell stage (Aoki et al. 1997; Schultz 1993). Hence, the first cleavages depend on maternal factors, while compaction and blastocyst formation depends on both maternal and embryonic factors. Over the past few years, some researchers focused on oocyte-restricted maternal effect gene because of their specific expression profiles (Dean 2002; Dharma et al. 2009; Evsikov et al. 2006; Paillisson et al. 2005; Yoon et al. 2006), which are mainly expressed in oocytes, remained present in zygotes and downregulated sharply after broad zygotic genome activation (Li et al. 2008; Ma et al. 2006; Ohsugi et al. 2008; Wu et al. 2003).

Mater (Maternal Antigen that Embryos Require) is one such oocyte-specific maternal effect gene and is required for early embryonic development in the mouse (Tong et al. 2000a, b, 2004; Tong & Nelson 1999). *Mater*, also known as *Nalp5*/*Nlrp5*, is a member of *Nlrp* gene family. *Nlrp14*, also known as *Nalp-iota*, another member of this family, is also essential for normal development of preimplantation embryos (Hamatani et al. 2004). Likewise, the latest evidence indicated that maternal depletion of *Nlrp2* in zygotes led to early embryonic arrest (Peng et al. 2012). Besides *Nlrp2*, *Nlrp5* and *Nlrp14*, several other *Nlrp* genes display specific or preferential oocyte expression patterns, the expression of which declines with oocyte aging (Dade et al. 2004; Hamatani et al. 2004).

Taken together, the limited previous studies indicate that this gene family may play a pivotal role in oogenesis and/or preimplantation embryo development. Thus, our group is interested in expression profiles of this gene family, and studied the tempo-

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ral and spatial expression patterns of *Nlrp9a* (also known as *Nalp9a*/*Nalp-theta*), *Nlrp9b* (also known as *Nalp9b*/*Nalp-delta*) and *Nlrp9c* (also known as *Nalp9c*/*Nalp-zeta*) in mouse development.

Material and methods

Animals

This study was approved by the Animal Care Commission of the College of Veterinary Medicine, Northwest A&F University. ICR strain mice were obtained from the Experimental Animal Center of The Fourth Military Medical University (Xi'an, China) and were maintained in accordance with the Animal Experiment Handbook at the Laboratory Animal Facility.

Collection of spermatozoa, oocytes and preimplantation embryos

Spermatozoa were collected from epididymides removed from mature 12-week-old ICR male mice.

Ovarian oocytes at the germinal vesicle (GV) stage were collected from ovaries removed from 4- to 40-week-old females injected with 10 IU of pregnant mare serum gonadotropin (PMSG) to stimulate the growth of the follicles. Forty-eight hours after PMSG administration the ovaries were removed and placed in Hepes-buffered KSOM medium (H-KSOM) (Biggers et al. 2000) containing 250 μM dibutyryl cyclic AMP to prevent spontaneous meiotic maturation. Oocytes were obtained from the largest follicles by puncturing them with a hypodermic needle. Superovulation was induced in female mice by injection of PMSG, followed 48 h later by injection of human chorionic gonadotropin (hCG). Metaphase II oocytes were collected from the oviduct ampullae in H-KSOM medium at 20 h after hCG injection. After the treatment of hyaluronidase (1 mg ml^{-1}), mature oocytes and cumulus cells were obtained respectively. Collection of preimplantation embryos was performed according to previously described protocols (Wang et al. 2008).

Cell culture

Cell lines (RAW264.7, Mouse D3 and F9 ES, EMT6) were cultured according to previously described protocols (Brandt 2010; Cho et al. 2009; Estes et al. 1997; Thompson & Gudas 2002). Cells were cultured at 37 °C in a humidified 5% CO_2/95% air incubator.

RNA isolation and cDNA preparation

Total RNA extracts from 2-week-old mouse tissues (ovary, uterus, testis, kidney, lung, heart, liver, brain, stomach, small intestine, muscle and spleen) were purified with RNeasy Mini Kits (Qiagen, Valencia, CA, USA). cDNA synthesis was performed using PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa, Otsu, Japan). Oocytes, preimplantation embryos and different cells (RAW264.7, D3 ES, F9 ES, EMT6, cumulus cells, oocytes and spermatozoa) were lysed and first-strand cDNA directly was synthesized using SuperScript®III CellsDirect cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. Lysis and reverse transcription were performed in the same tube. DNase I was added to eliminate genomic DNA prior to first strand synthesis.

Table 1. Primer sequences for RT–PCR and quantitative real-time PCR.

Genes	Primer sequences (5′–3′)	T^a_{ann} (°C)
Nlrp9a	F[b]: GACTTCACCAGTGACTGTTGTG	56
	R[c]: CCATACCAGACGAACACCC	
Nlrp9b	F: CACCAGTGACTGTTGTAAGGAC	57
	R: TTCCTGCTGTTCCATACCAG	
Nlrp9c	F: TTCCAGTGAATCAACATCAGCT	60
	R: GAACAGGGTAGCAAAGTACCA	
β-actin	F: GAAGTGTGACGTTGACATCCG	60
	R: ACTTGCGGTGCACGATGGAGG	

Explanations: [a] Annealing temperature. [b] Forward primer. [c] Reverse primer.

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

The primer pairs used for RT–PCR and qRT–PCR and their annealing temperatures are described in Table 1. cDNA samples were amplified by PCR with Ex Taq DNA polymerase (TaKaRa). Reaction mixtures contained 1 × Ex Taq buffer, 2.5 mM dNTP, 40 pmol of primers, and 1.25 units of Ex Taq in a final reaction volume of 50 μl. PCR conditions were as follows: 95 °C for 1 min; 94 °C for 30 s, 56–60 °C (Table 1) for 30 s, 72 °C for 25 s for 35 cycles, and 72 °C for 6 min. The mRNA levels were quantified using SYBR Premix Ex Taq™ II (TaKaRa) on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Inc., Carlsbad, CA, USA). Thermal cycling conditions were 95 °C for 1 min, followed by 40 PCR cycles of 5 s at 95 °C for DNA denaturation and 30 s at 56–60 °C, 25 s at 72 °C for primer annealing and extension. Transcripts of each gene were quantified in three replicates. Transcript levels were calculated relative to the transcription of the housekeeping gene β-actin in every sample. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was chosen to calculate the relative mRNA levels.

In situ hybridisation

Nlrp9a, *Nlrp9b* and *Nlrp9c* mRNAs were detected using ISH Detection Kits (Boster-Bio, China) according to the manufacturer's instructions. The probes used for ISH are described in Table 2. ISH analysis was performed on paraffin sections of mouse ovaries obtained from 4-week-old mice. After deparaffinisation and rehydration, sections were treated with proteinase K for 30 s at room temperature. After washing with PBS, sections were refixed with 1% paraformaldehyde. Each section was prehybridised and hybridized in a moist chamber. Hybridized sections were washed twice with 2× saline sodium citrate (SSC) buffer for 10 min, three times 0.5× SSC for 15 min and twice in 0.2× SSC for 20 min at 37 °C. Hybridised probes were detected immunohistochemically by incubating the sections with biotinylated anti-DIG antibody and then detecting the probes with streptavidin alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium substrate solution according to the manufacturer's instructions. Negative controls consisted of identical reactions with sense probes.

Statistical analysis

Each experiment was repeated at least three times and data were presented as the mean ± SEM.

Table 2. Probe sequences for ISH.

Genes	Probe sequences (Antisence)
Nlrp9a	5′–GGAGTCCTAATGTTCGACCAAAACGACTTGCTACCTCTCTCCTCTCAT–3′
Nlrp9b	5′–GTATTTCGTCTTTTGCCTATAGAAGTTCTTGTTTTACAATGGTCTGGG–3′
Nlrp9c	5′–GCTGATGTTGATTCACTGGAAGAGATTACTTCCTCTCTCGAATAATGC–3′

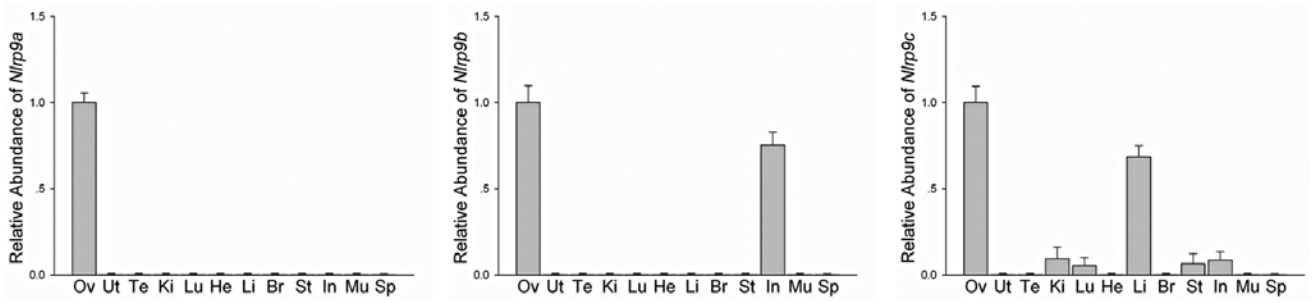

Fig. 1. Analysis of *Nlrp9a*, *Nlrp9b* and *Nlrp9c* expression in mouse tissues by qRT-PCR with total RNA extracted from 2-week-old mouse ovary (Ov), uterus (Ut), testis (Te), kidney (Ki), lung (Lu), heart (He), liver (Li), brain (Br), stomach (St), intestines (In), muscle (Mu), spleen (Sp) were performed. Results were normalized to the abundance in the ovary and expressed as the mean ± SEM.

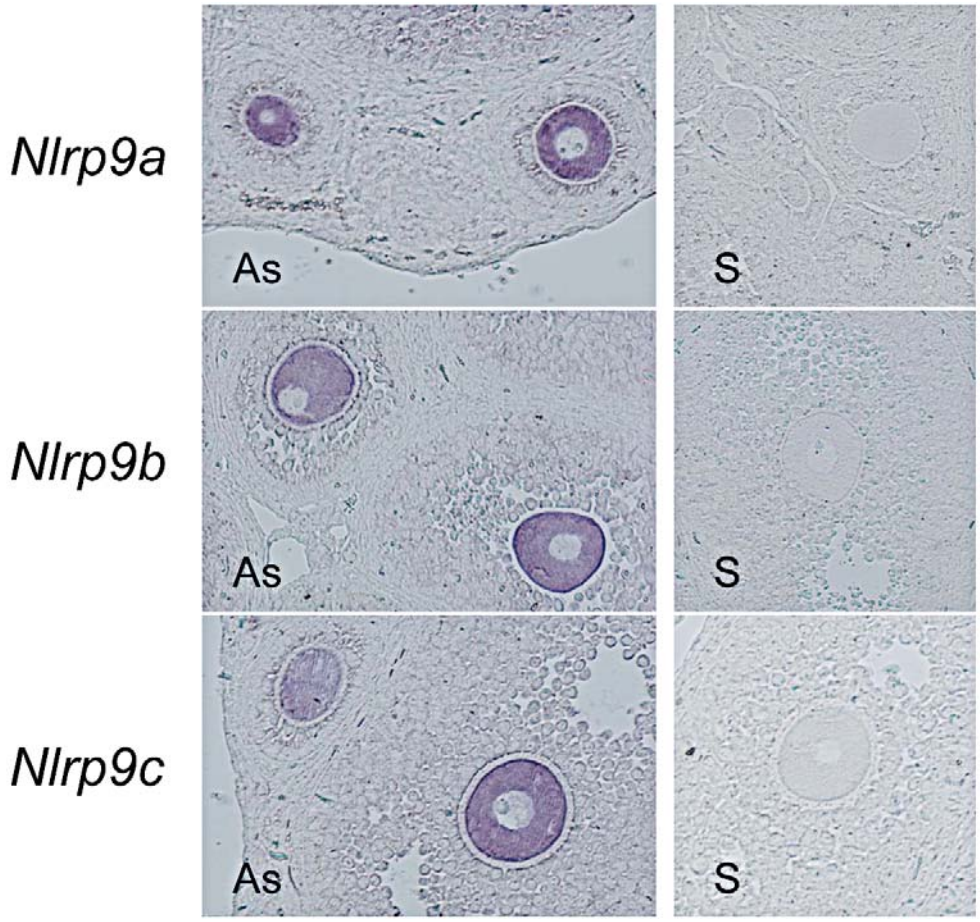

Fig. 2. Expression analysis of *Nlrp9a–Nlrp9c* in the ovary. ISH of ovarian sections probed with digoxigenin (DIG)-labelled oligonucleotide antisense (As) and sense (S) probes. (Original magnification ×100)

Results

Expression of Nlrp9a, Nlrp9b and Nlrp9c in mouse tissues

Relative expression levels of *Nlrp9a*, *Nlrp9b* and *Nlrp9c* were analyzed in twelve tissues in the mouse using qRT-PCR. *Nlrp9a* expression was detected in mouse ovary but not in eleven other tissues including male testis. Expression of *Nlrp9b* was also detected in mouse ovary, as well as in intestine. qRT-PCR analysis revealed that

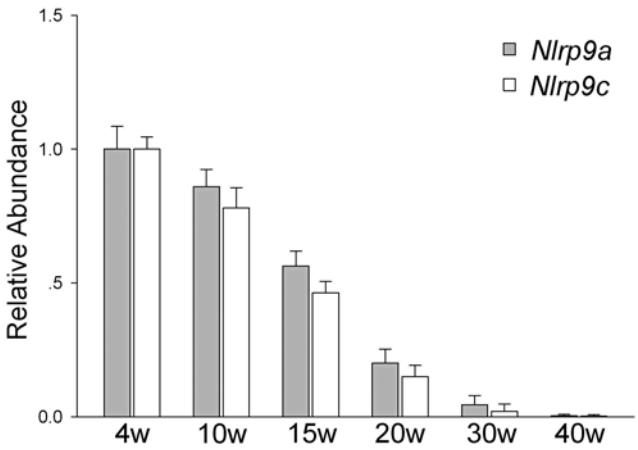

Fig. 3. Expression analysis of *Nlrp9a* and *Nlrp9c* in GV-stage oocytes obtained from mouse ovaries at different ages. qRT-PCR of *Nlrp9a* and *Nlrp9c* expression using total RNA isolated from GV-stage oocytes obtained from mouse ovaries at 4, 10, 15, 20, 30 and 40 weeks (w). Results were normalized to the abundance in 4-w-old group and expressed as the mean ± SEM.

Nlrp9c was expressed at high levels in mouse ovary and liver, with detectable expression also observed in intestine, stomach, kidney and lung (Fig. 1).

Expression of Nlrp9a, Nlrp9b and Nlrp9c in mouse ovaries

To further determine the expression of *Nlrp9a*, *Nlrp9b* and *Nlrp9c* in mouse ovaries, ISH was performed. Analysis of ovarian sections by ISH suggested that *Nlrp9a*, *Nlrp9b* and *Nlrp9c* transcripts were detected exclusively in oocytes within the ovary (Fig. 2).

Expression of Nlrp9a and Nlrp9c declines in ovaries with mouse aging

There was a literature showed that down-regulation of *Nlrp9b* expression in ovaries was connected with mouse aging (Hamatani et al. 2004). To determine if the expressions of *Nlrp9a* and *Nlrp9c* would connect with mouse aging, we investigated the expression levels of these genes in GV-stage oocytes obtained from mouse ovaries at the different ages. We found that the expression of these transcripts was declined with mouse aging (Fig. 3).

Expression of Nlrp9a, Nlrp9b and Nlrp9c during preimplantation embryo development

To determine the expression patterns of *Nlrp9a*, *Nlrp9b* and *Nlrp9c*, we performed RT-PCR analyses. As shown in Fig. 4, *Nlrp9a*, *Nlrp9b* and *Nlrp9c* have the identical expression patterns during preimplantation development. The transcripts of *Nlrp9a*, *Nlrp9b* and *Nlrp9c* were all detected in MII oocytes, zygotes and 2-cell embryos. However, the abundance of these transcripts in 2-cell embryos was lower than the abundance in MII oocytes and zygotes. These transcripts were not detected from 4-cell embryos to blastocysts.

Expression of Nlrp9a, Nlrp9b and Nlrp9c in mouse cells

To confirm whether *Nlrp9a*, *Nlrp9b* and *Nlrp9c* would express in different cells, we performed RT-PCR analyses. *Nlrp9a* and *Nlrp9c* expressions were detected in mouse oocytes but not in other cells. *Nlrp9b* expression was also detected in oocytes, with detectable expression observed in D3 ES and F9 ES (Fig. 5).

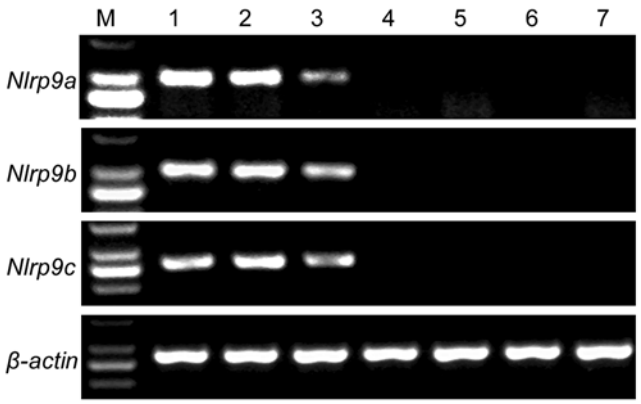

Fig. 4. Expression analysis of *Nlrp9a–Nlrp9c* in oocytes and preimplantation embryos by RT-PCR using cDNA synthesized from mature oocytes, 1-cell, 2-cell, 4-cell, 8-cell embryos, morula and blastocyst (Lanes 1–7, respectively). *β*-actin was used as a control.

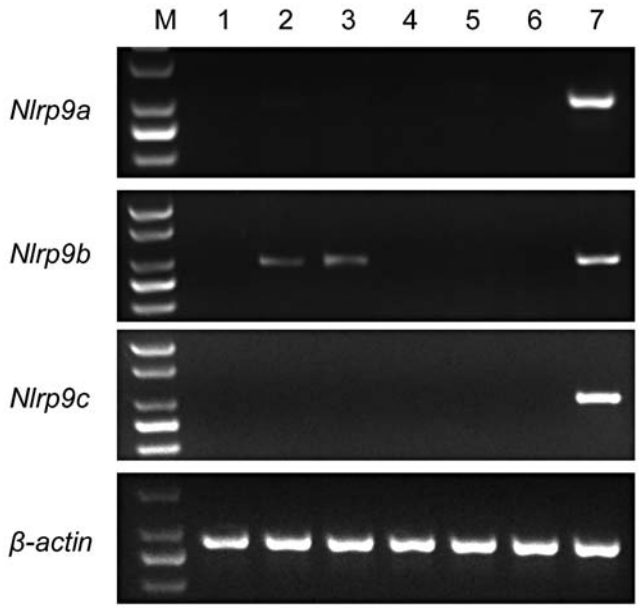

Fig. 5. Analysis of *Nlrp9a*, *Nlrp9b* and *Nlrp9c* expression in different mouse cells. RT-PCR of *Nlrp9a*, *Nlrp9b* and *Nlrp9c* expression using total RNA isolated from RAW264.7, D3 ES, F9 ES, EMT6, cumulus cells, spermatozoa and oocytes (Lanes 1–7, respectively). *β*-actin was used as a control.

Discussion

The *NLRP* gene family contains 14 members in rhesus macaque monkeys and humans (McDaniel & Wu 2009; Zhang et al. 2008), while 20 *Nlrp* family members have been identified in the mouse. Because three *Nlrp* genes show lineage-specific duplications including *Nlrp1* (*Nlrp1a, Nlrp1b* and *Nlrp1c*), *Nlrp4* (*Nlrp4a-*

Nlrp4g) and *Nlrp9* (*Nlrp9a, Nlrp9b* and *Nlrp9c*), meanwhile, *Nlrp7*, *Nlrp8*, *Nlrp11* and *Nlrp13* are lost in the mouse. The phylogenetic analyses identified a well supported reproduction-related clade including nine NLRP proteins (NLRP2, 4, 5, 7, 8, 9, 11, 13 and 14) in humans (Tian et al. 2009). In the reproduction-related *NLRP* cluster, there are several oocyte-specific *NLRP* genes including *NLRP2*, *4*, *5*, *8*, *9* and *14* (Tian et al. 2009). *Nlrp8* is lost, while *Nlrp2*, *Nlrp5* (*Mater*) and *Nlrp14* have been investigated and showed that these genes are required for early embryonic development in the mouse (Hamatani et al. 2004; Peng et al. 2012; Tong et al. 2000a). Thus, we selected and investigated the temporal and spatial patterns of *Nlrp9* expression in this study to pave the way for further functional studies in the mouse.

The previous studies showed that *Nlrp9a*, *Nlrp9b* and *Nlrp9c* were expressed in mouse ovaries (Dade et al. 2004; Hamatani et al. 2004). However, our further research demonstrated that expression of *Nlrp9b* and *Nlrp9c* was detected not just in ovaries, but in other tissues. These results suggest that *Nlrp9b* and *Nlrp9c* might involve in oogenesis or organogenesis. Meanwhile, the expression of *Nlrp9a*, *Nlrp9b* and *Nlrp9c* is restricted to the oocytes within the ovary. These expression patterns are identical to that of *Nlrp5* in ovaries from the mouse (Tong et al. 2000, 2004), bovine (Pennetier et al. 2006; Ponsuksili et al. 2006), pig (Pisani et al. 2010), rhesus macaque (McDaniel & Wu 2009; Wu 2009) and human (Tong et al. 2002).

It has been shown that down-regulation of *Nlrp9b* expression in ovaries was connected with mouse aging (Hamatani et al. 2004). Our investigation also validated that *Nlrp9a* and *Nlrp9c* transcripts were also declined with mouse aging, suggesting that *Nlrp9a*, *Nlrp9b* and *Nlrp9c* are involve in female reproduction and might play a role during oocyte development. Further research of the function of these genes could provide insights into the phenomenon that female fertility declines with aging.

The expression patterns of *Nlrp9a*, *Nlrp9b* and *Nlrp9c* revealed that the source of embryonic *Nlrp9a*, *Nlrp9b* and *Nlrp9c* transcripts are maternal and do not come from expression of the zygotic genome during preimplantation development, implying that these genes might play an essential role in early embryo development. Hence, further functional studies such as knock-out models or other targeted inhibition experiments on these genes may confirm this hypothesis. In addition, *Nlrp9a* and *Nlrp9c* transcripts were not detected in other cells except for oocytes. Nevertheless, *Nlrp9b* expression was detected in oocytes, as well as in D3 ES and F9 ES, implying that *Nlrp9b* might also play a role in ES.

There is a different expression profile of *Nlrp9* (*NALP9*/*NLRP9*) during preimplantation development in mammals and primates. *NALP9* is characterized as a novel oocyte marker gene and detected in ovary and testis (Dalbies-Tran et al. 2005; Ponsuksili et al. 2006), after fertilization, *NALP9* expression is declined and disappears with zygotic genome activation in cattle (Ponsuksili et al. 2006), as well as in the mouse. However, *NLRP9* transcripts are detected in oocytes and zygotes, this expression remains present through the blastocyst stage in rhesus macaque monkeys (McDaniel & Wu 2009), as well as in humans (Zhang et al. 2008). These researches have shown that different lineages adapted to develop their own *Nlrp* (*NLRP*) genes during evolution, with functionally diversified in mammalian reproductive systems.

In summary, the present study investigated the expression patterns of *Nlrp9a*, *Nlrp9b* and *Nlrp9c*, which were all strikingly expressed in the ovary. The transcripts of *Nlrp9a*, *Nlrp9b* and *Nlrp9c* were restricted to the oocytes and declined with oocyte aging within the ovary, which display specific or preferential oocyte expression patterns. Meanwhile, the three *Nlrp9* transcripts were maternal and did not come from expression of the zygotic genome during preimplantation development. In addition, *Nlrp9a* and *Nlrp9c* transcripts were only detected in oocytes, while *Nlrp9b* transcripts were detected not only in oocytes, but also in D3 and F9 ES. Thus, the study revealed the important roles of the three *Nlrp9* genes in oogenesis, oocyte maturation and early embryo development in the mouse, research into the function of these genes will shed light on the molecular basis underlying oogenesis and/or preimplantation development.

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