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Expression Analysis of Nlrp4a-Nlrp4f During Mouse Development

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Abstract: The *Nlrp* gene family plays an essential role in the innate immune and reproductive systems in the mouse. Initially, studies on the function of this family were mainly in apoptotic and inflammatory signaling pathways. However, a rapidly growing number of recent researches showed that some *Nlrp* genes play key roles in reproductive systems. In this study, researchers investigated the expression patterns of Nlrp4a-Nlrp4f during mouse development. The results showed that these genes have the similar expression patterns during preimplantation development. They were enriched in the GV stage and MII oocytes and degraded after fertilization but Nlrp4c and Nlrp4e transcripts were detected again at the morula and blastocyst stages. The tissue distribution of Nlrp4a-Nlrp4f indicated that Nlrp4b and Nlrp4c were only detected in the ovary; Nlrp4a, Nlrp4d and Nlrp4f were also transcribed in the ovary as well as in the testis while Nlrp4e was expressed in various mouse tissues. Furthermore, expression of Nlrp4b-Nlrp4e was downregulated in the ovary with mouse aging. In addition, the expression profiles of Nlrp4a-Nlrp4f in different cells demonstrated that Nlrp4a, Nlrp4b, Nlrp4c, Nlrp4e and Nlrp4f were not detected in other cell lines except for oocytes while Nlrp4d transcripts were detected in oocytes as well as in cumulus cells and spermatozoa. The results indicated that Nlrp4a-Nlrp4f displays specific or preferential oocyte expression patterns, implying important roles of these genes in oogenesis and preimplantation development in the mouse.

Key words: Expression analysis, Nlrp4, mouse, spermatozoa, cumulus cell

INTRODUCTION

The *Nlrp* (*NLRP*) gene family plays an essential role in the innate immune and reproductive systems in the mouse and primates. Initially, studies on the function of this family were mainly in apoptotic and inflammatory signaling pathways (Reik and Maher, 1997; Hoffman *et al*., 2001; Bouchier-Hayes *et al*., 2001; Chu *et al*., 2001; Manji *et al*., 2002; Wang *et al*., 2002). However, a rapidly growing number of recent researches showed that some *Nlrp* (*NLRP*) genes play key roles in reproductive systems (Tong *et al*., 2000; Hamatani *et al*., 2004b; Murdoch *et al*., 2006; Zhang *et al*., 2008; McDaniel and Wu, 2009; Peng *et al*., 2012). For example, maternal knockout/depletion of Nlrp5 (Mater)/NLRP5 blocks early embryogenesis in the mouse and rhesus macaque monkeys (Tong *et al*., 2000; Wu, 2009).

Germline mutations in NLRP2 result in a familial imprinting disorder (Beckwith-Wiedemann Syndrome) in humans (Meyer *et al*., 2009) while the mutations of NLRP7 are found to cause recurrent hydatidiform moles (Murdoch *et al*., 2006; Qian *et al*., 2007). Moreover, knockdown/mutation of *Nlrp14*/*NLRP14* gene leads to early embryogenesis and spermatogenic failure in the mouse and humans (Hamatani *et al*., 2004b; Westerveld *et al*., 2006), respectively. Researches indicated that most *NLRP* genes are expressed in primate gametes and early embryos (Zhang *et al*., 2008; McDaniel and Wu, 2009; Peng *et al*., 2013) in which contain several oocyte-specific *NLRP* genes including *NLRP4*, *5*, *8*, *9* and *14* (Tian *et al*., 2009). Nlrp8 is lost in the mouse while Nlrp5 (Mater) and Nlrp14 have been investigated and showed that these genes are required for early embryonic development (Tong *et al*., 2000; Hamatani *et al*., 2004b). *Nlrp4* gene show lineage-specific duplications in the mouse. However, the expression patterns of these genes have not been reported. Thus, researchers selected and investigated the expression patterns of Nlrp4a-Nlrp4f in this study to pave the way for further functional studies in the mouse.

MATERIALS AND METHODS

Animals: Adult male and female ICR strain mice were purchased from the Experimental Animal Center of Fujian Medical University (Fuzhou, China). The experimental procedure was approved by the Animal Care Commission of College of Animal Science, Fujian Agriculture and Forestry University.

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Chemicals and reagents: 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 of oocytes and preimplantation embryos: ICR strain female mice 4-40 weeks old of age was superovulated by intraperitoneal injections of 10 international units of Pregnant Mare Serum Gonadotrophin (PMSG) to stimulate the growth of the follicles. The 48 h after PMSG administration the ovaries were placed in Hepes-buffered KSOM medium (H-KSOM) (Biggers *et al*., 2000) containing 250 μM dibutyryl cyclic AMP to inhibit resumption of meiosis and immature oocytes displaying a GV were released from the largest follicles by puncturing them with hypodermic needle. Metaphase II oocytes were collected from the oviduct ampullae in H-KSOM at 20 h after the hCG injection. Cumulus masses were treated with hyaluronidase (1 mg mL^{-1}) to release ova. Metaphase II oocytes and cumulus cells were collected separately. To obtain preimplantation embryos, females were mated overnight with males and checked for vaginal plugs the next morning. Collection of preimplantation embryos was performed according to previously described protocols (Wang *et al*., 2008).

Collection of spermatozoa: Epididymides were removed from mature 12 weeks 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 medium was added to each tube. Then, the tube was centrifuged (3000 rpm, 3 min) to collect spermatozoa.

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

RNA isolation and cDNA preparation: 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. Total RNA extracts from 2 weeks 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).

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

Genes	Primer sequences (5'-3')	T_{ann}^{a} (°C)
Nlrp4a	F[b]: ACAATGGGTTGGTTGTCCTGTG	60
	R[c]: CCAGAAATGCCTGGGTTTCAGTA	
Nlrp4b	F: GAGGATCAACCTGGCGAAGA	60
	R: TCCTGGGAATCGCTATCAAAGTC	
Nlrp4c	F: CACCCAGACTGCGTTCTGAAG	60
	R: TTCAGGTCATTGGAGCTGATGTCTA	
Nlrp4d	F: TGAGGGTATGTGGACTGG	60
	R: GGACAGATGGGTGGAGGAATA	
Nlrp4e	F: AGGCTTTGTGCCACCCAGA	60
	R: CTCACTGAGGGAACATTTGGCTA	
Nlrp4f	F: CTTGAACCAGGCAGAGTGCAAC	60
	R: TGCCAAATTAAGAACCTTCAACGAC	
β-actin	F: GAAGTGTGACGTTGACATCCG	60
	R: ACTTGCGGTGCACGATGGAGG	

[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. Each primer pair was validated by performing electrophoresis and melting temperature analysis of the PCR product to ensure the correct size of PCR product and the absence of primer dimers. RT-PCR was performed using 35 cycles of 94°C for 30 sec, annealing temperature for 30 sec and elongation at 72°C for 1 min kb^{-1}. Reactions were performed using 1.25 units ExTaq DNA polymerase (TaKaRa), 1×ExTaq buffer, 2.5 mM dNTP and 40 pmol of primers in a final reaction volume of 50 μL. The mRNA levels were quantified using SYBR Premix ExTaq™ II (TaKaRa) on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Inc., Carlsbad, CA, USA). Samples were denatured at 95°C, 1 min and then subjected to 40 cycles of amplification (95°C, 5 sec; 60°C, 30 sec). Standard concentration curves were done for each primer pair used. Each data point was the average of duplicate assays performed on three independently obtained samples and transcript levels were calculated relative to the transcription of the housekeeping gene *β-actin* in every sample. Fold changes for each gene were calculated using the $2^{-\Delta\Delta CT}$ Method (Livak and Schmittgen, 2001).

Statistical analysis: Each experiment was repeated at least three times and results were presented as the mean±SEM.

RESULTS AND DISCUSSION

Expression of Nlrp4a-Nlrp4f during preimplantation embryo development: To elucidate the temporal expression of Nlrp4a-Nlrp4f during preimplantation embryo development, researchers conducted RT-PCR analyses. As shown in Fig. 1, Nlrp4b, Nlrp4d and Nlrp4f have the similar expression patterns during preimplantation development. These genes examined were highly expressed in fully grown GV-intact and Mature (MII) oocytes. After fertilization, they were immediately downregulated and not detected after the 2-cell stage.

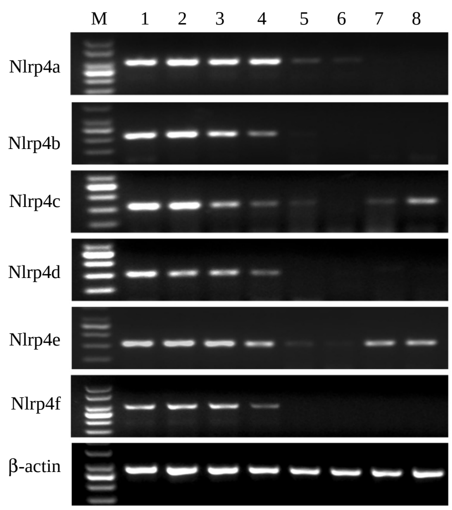

Fig. 1: Expression analysis of Nlrp4a-Nlrp4f in oocytes and preimplantation embryos by RT-PCR using cDNA synthesized from GV-stage oocytes, mature oocytes, 1-cell, 2-cells, 4-cells, 8-cells embryos, morula and blastocyst (Lanes 1-8, respectively). β-actin was used as a control

While Nlrp4a was expressed at high levels before the 2-cell stage with detectable expression also observed at the 4-cell and 8-cell stages. Nlrp4c and Nlrp4e transcripts were enriched in the GV stage and MII oocytes and degraded after fertilization but they were detected again at the morula and blastocyst stages.

Tissue distribution of mouse Nlrp4a-Nlrp4f: To determine the tissue distribution of Nlrp4a-Nlrp4f, researchers performed qRT-PCR analyses in twelve mouse tissues. As shown in Fig. 2, Nlrp4b and Nlrp4c were only detected in the ovary while Nlrp4a, Nlrp4d and Nlrp4f were also transcribed in the ovary as well as in the testis. Nlrp4e was highly expressed in the ovary, liver, stomach and intestines with detectable expression also observed in the testis, kidney, lung and heart.

Down-regulation of Nlrp4b-Nlrp4e expression in ovaries with mouse aging: Some literature showed that expression of Nlrp4a and Nlrp4f was downregulated in the ovary with mouse aging (Hamatani *et al*., 2004b). Thus, to determine if expression of other *Nlrp4* genes was connected with mouse aging, we investigated the expression profiles of these genes in GV-stage oocytes obtained from mouse ovaries at the different ages. As shown in Fig. 3, the transcripts of other *Nlrp4* genes were declined with mouse aging.

Expression of Nlrp4a-Nlrp4f in mouse cells: To investigate the expression profiles of Nlrp4a-Nlrp4f in

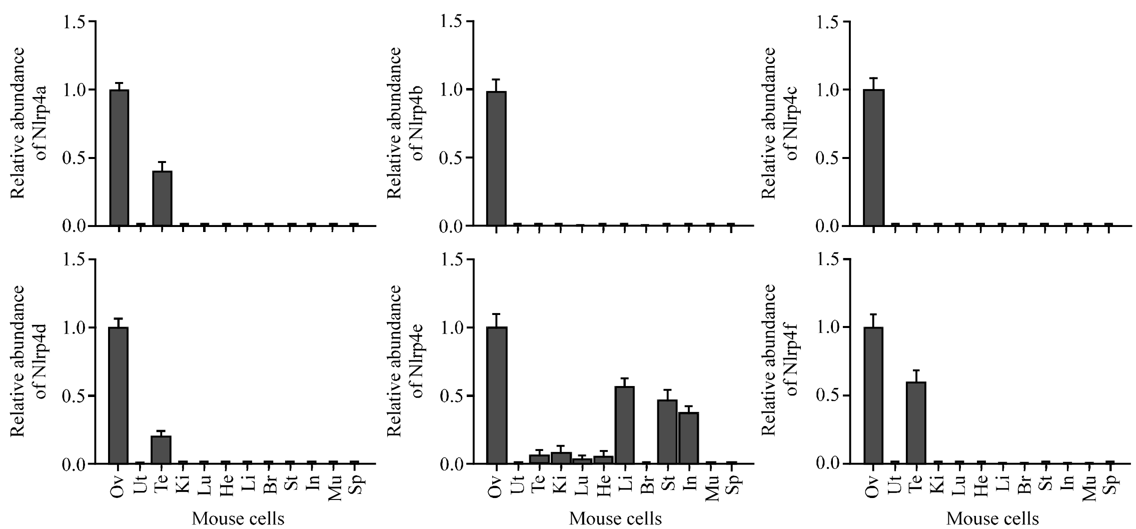

Fig. 2: Analysis of Nlrp4a-Nlrp4f expression in mouse tissues by qRT-PCR with total RNA extracted from 2 weeks old mouse Ovary (Ov), Uterus (Ut), Testis (Te), Kidney (Ki), Lung (Lu), Heart (He), Liver (Li), Brain (Br), Stomach (St), Intestines (In), Muscle (Mu), Spleen (Sp) were performed. Results were normalized to the abundance in the ovary and expressed as the mean±SEM

different cells, qRT-PCR was carried out. As shown in Fig. 4, these genes have the similar expression profiles except for Nlrp4d. They were all expressed in oocytes, while Nlrp4d transcripts were detected in oocytes as well as in cumulus cells and spermatozoa.

The expression profiling of Nlrp4a-Nlrp4f during preimplantation embryo development was concordant with the earlier microarray experiment (Hamatani *et al*., 2004a). These genes were expressed in oocytes but were immediately downregulated after fertilization. It indicated that these transcripts are present with the exclusive maternal origin during mouse preimplantation stages implying that these genes might play a role in preimplantation embryo development. To research this possibility, further functional studies such as knock-out models or other target edinhibition experiments on these genes should be performed. *Nlrp4* genes show lineage-specific duplications during evolution in the mouse and rat while *NLRP4* gene is only one in primates and humans. It has been shown that the relatively high-level expression of NLRP4 persists until the 4-cell stage and diminishes thereafter with detectable expression also observed at the morula and blastocyst stages in rhesus macaque monkeys (McDaniel and Wu, 2009) as well as in humans (Zhang *et al*., 2008). Thus, the expression profiling of *NLRP4* gene in primates and humans is similar to the profiling of Nlrp4c and Nlrp4e in the mouse.

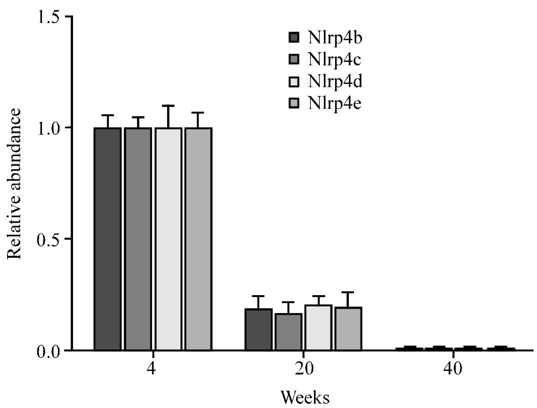

Fig. 3: Expression analysis of Nlrp4b-Nlrp4e in GV-stage oocytes obtained from mouse ovaries at different ages. qRT-PCR of Nlrp4b-Nlrp4e expression using total RNA isolated from GV-stage oocytes obtained from mouse ovaries at 4, 20 and 40 weeks. Results were normalized to the abundance in 4 weeks old group and expressed as the mean±SEM

The expression pattern of Nlrp4a, Nlrp4b and Nlrp4c in mouse tissues was consistent with earlier reports (Hamatani *et al*., 2004b; Dade *et al*., 2004). But tissue distribution of Nlrp4f was different, it was also found expressed in the testis. Nlrp4a, Nlrp4d and Nlrp4f show homology to rhesus macaque NLRP4 as they were all relatively enriched in both the ovary and testis (McDaniel and Wu, 2009). Interestingly, these *Nlrp4*

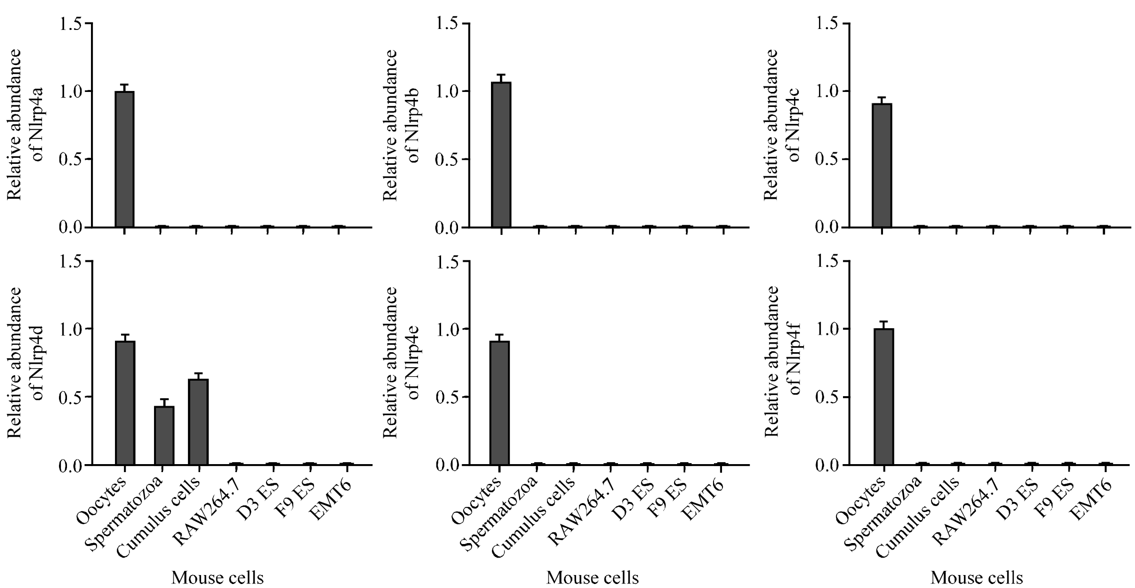

Fig. 4: Analysis of Nlrp4a-Nlrp4f expression in different mouse cells. qRT-PCR of Nlrp4a-Nlrp4f expression using cDNA synthesized from oocytes, spermatozoa, cumulus cells, RAW264.7, D3 ES, F9 ES and EMT6. Results were normalized to the abundance in the oocytes and expressed as the mean±SEM

genes have the common characteristic that they were all expressed in the ovary. The expression pattern of these genes suggests important roles of these genes in the reproductive system in the mouse.

Several *Nlrp* genes have been found to be related to reproduction in the mouse (Tong *et al*., 2000; Hamatani *et al*., 2004b; Dade *et al*., 2004; Horikawa *et al*., 2005; Evsikov *et al*., 2006). The results also demonstrated that *Nlrp4* genes associated with mouse reproduction. Perhaps the decline of reproduction-related genes in oocytes with female aging leads to the lower developmental competence in oocytes or early embryos and further displays female subfertility or infertility. Thus, research into the function of these genes and the mechanisms controlling their expression will shed light on the molecular basis underlying female subfertility or infertility.

Some *Nlrp* genes play an essential role in the innate immune system (Kanneganti *et al*., 2006; Boyden and Dietrich, 2006; Gross *et al*., 2009); they were all expressed in this system in the mouse (Guarda *et al*., 2009; Nakahira *et al*., 2011). However, Nlrp4a-Nlrp4f was not detected in the spleen and RAW264.7 murine macrophage-like cells indicating that these genes were not involved in the innate immune system in the mouse. Interestingly, they were all expressed in oocytes, demonstrating again that these genes associated with mouse reproduction. The expression pattern of Nlrp4a-Nlrp4f was consistent with the expression of NLRP4 in rhesus macaque monkeys (McDaniel and Wu, 2009).

CONCLUSION

In summary, researchers investigated the patterns of Nlrp4a-Nlrp4f during mouse development. The expression of Nlrp4a-Nlrp4f indicated that these genes are oocyte-selective, implying important roles of these genes in oogenesis and preimplantation development in the mouse. Thus, research into the function of these genes and the mechanisms controlling their expression will shed light on the molecular basis underlying oogenesis and preimplantation embryonic development.

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