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—Full Paper—

Characterization of Maternal Antigen That Embryos Require (MATER/NLRP5) Gene and Protein in Pig Somatic Tissues and Germ Cells

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Abstract. Maternal effect genes produce mRNA or proteins that accumulate in the egg during oogenesis and control the developmental program until embryonic genome activation takes place. NLRP5 (NLR family, Pyrin domain containing 5), also called MATER (Maternal Antigen That Embryos Require) is one of the genes required for normal early embryonic development, although its precise function remains to be elucidated. The aim of the present study was to analyze the NLRP5 gene expression pattern and protein distribution in somatic tissues and germ cells in the pig. Reverse transcription was performed on mRNA from germinal vescicle (GV) oocytes and total RNA from spermatozoa and tissues from different organs. The transcript for NLRP5 gene was identified only in ovaries and oocytes. The presence of NLRP5 protein was detected only in ovaries by western blot analysis and immunohistochemistry.
Key words: Maternal effect genes, NLR family, Pyrin domain containing 5 (NLRP5), Oocytes, Pig

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Over the past few years, a number of mammalian genes predominantly or exclusively expressed in germ cells have been discovered. Functional studies have reported the essential role of these genes during gametogenesis, folliculogenesis and early embryonic development [1–7]. NLRP5 (NLR, pyrin domain containing5), also called MATER (Maternal Antigen That Embryos Require), is one of these genes. However, its precise function remains to be elucidated, although the transcription patterns may suggest a role in embryonic genome activation.

Maternal mRNAs accumulated in the oocyte have a crucial role for successful embryo development before activation of the embryonic genome (EGA) [3], and some of them are also required for embryo development after EGA and implantation. These genes, which are called maternal effect genes and which include NLRP5, Zygotic Arrest 1 (Zar1) [8, 9], Stella [10, 11] and Nucleoplasmin 2 (Npm2) [12], are all required for normal embryonic development beyond the 1- and 2-cell stage in mice [13, 14]. Knock-out of these genes leads to incapacity of the embryo to develop beyond the 2-cell stage [15].

Developmental block in embryos produced *in vitro* remains the main problem in assisted reproduction of farm animals, particularly in cattle and pigs. An increasing amount of data indicates that the embryonic genome activation is crucial for the success of preimplantation embryo development [16]. To ensure maternal-embryo transition (MET) of gene expression, oocytes have to reach a sufficiently advanced level of developmental competence during differentiation and maturation [17–19]. Maternally expressed genes are implicated in this process, and some of them are associated with developmental competence [20, 21]. In domestic species, MET occurs at the 4–16-cell stage [22], a stage in which enough biological material is available to study the fate of maternal mRNAs and their action in regulating embryo cleavages. Studying these genes in farm animals provides a model for understanding the mechanisms that affect oocyte quality and their implication in the success of embryo development and survival.

NLRP5 is an oocyte-specific maternal effect gene first identified in the mouse, an animal in which it affects development beyond the 2-cell stage [15, 23]. More recently, the RNA and protein were observed up to the 16-cell stage in bovine embryos [24, 25], the 8-cell stage in a non-human primate [26] and in germinal vesicles in humans [27]. The transcript can be detected during oocyte growth from primary follicles, accumulates during oocyte development and decreases after fertilization [28, 29]. NLRP5-null female mice present a normal phenotype regarding folliculogenesis, ovulation and fertilization; however, their embryos do not develop beyond the 2-cell stage coincident with maternal-to-embryo transition [15]. The NLRP family is a subfamily of the newly described CATERPILLER (CAspase-recruiment domain (CARD) Transcription Enhancer, R (purine)-binding, Pyrin, Lots of LEucine Repeats) family, which comprises proteins with a nucleotide-binding domain and a leucine-rich domain [30]. In humans, the NLRP gene family is constituted of 14 members [31]. As these genes have only been discovered recently, information about their expression and functions in farm animals is still very limited.

The aim of this study was the identification and characterization in a second farm species, swine, of one of these genes, NLRP5, an oocyte-selective factor that may play an important role in oogenesis

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Table 1. Primer pairs used for correct mRNA retro-transcription and cDNA amplification

	Forward 5'–3'	Reverse 5'–3'	Amplicon size	Annealing temperature
Beta actin	aatcctgcggcatccacgaaacta	agaagcatttgcggtggacgat	318 bp	60 C
Histone H2AFZ	aggacgactagccatggacgtgtg	ccaccaccagcaattgtagccttg	208 bp	60 C
cds1	tccaattacggactgcagtggt	gctcttcgagtccatctaccacaa	772 bp	59 C
cds2	ggctcttgattgtggtagatggac	cgtagaacaaggccgcaaagaa	726 bp	60 C
cds3	aggagttctttgcggccttgtt	acccgggccccttttaaatatcaga	697 bp	59 C
cds4	ggcagccagcctgtaaaatacaga	tcgtgatcacgttggacagact	771 bp	59 C
cds5	agagtctgtccaacgtgatcacga	taggacagcgcttcacagagaac	637 bp	60 C

and preimplantation embryo development, thus confirming the data produced so far only in bovine.

Materials and Methods

Oocyte collection

Ovaries from puberal gilts were collected from a local abattoir and transported to the laboratory at 25–30 C in 0.9% saline. Cumulus oocyte complexes (COCs) were isolated under a stereomicroscope, recovered from each dish and transferred to a Petri dish containing Tissue Culture Medium 199 (TCM 199). Cumulus cells were removed using 3 mg/ml hyaluronidase in saline and repeated pipetting. Oocytes were collected in pools of 10 in 3 μl of washing medium, TCM 199, and stored at –80 C until use.

RNA isolation from oocytes

Extraction of mRNA from oocytes was performed using a Dynabeads mRNA DIRECT Micro-Kit (Invitrogen, Italy) according to the manufacturer's instructions. Briefly, samples were lysed in 100 μl of Lysis Buffer for 10 min at room temperature. Each sample was transferred into a clean tube with 2μl of Dynabeads Oligo$(dT)_{25}$ and gently mixed for 5 min. After two washes in Washing Solution A (10 mmol Tris-HCl, pH 8.0, 0.15 mmol LiCl, 1 mmol EDTA, 0.1% (w/v) SDS) and three washes in Washing Solution B (10 mmol Tris-HCl, pH 8.0, 0.15 mmol LiCl, 1 mmol EDTA), Poly$(A)^+$RNAs were eluted from the beads by incubation in 8.5 μl Diethylpyrocarbonate (DEPC)-treated water at 65 C for 2 min. Aliquots were immediately used for reverse transcription.

Tissue and spermatozoa collection and RNA extraction

Porcine tissues, which for these experiments included sample of the heart, liver, ovary, lung, spleen, medulla oblongata, tongue, muscle, subcutaneous adipose tissue and aorta, were collected at a local abattoir and transported to the laboratory in phosphate buffered saline (PBS), washed in fresh PBS and stored at –80 C. Porcine sperm samples were purified by centrifugation through a discontinuous Percoll density gradient (95:45, v/v) and swim-up technique. Pellets were gently overlaid with 1.2 ml PBS. Tubes were placed in an incubator at 39 C for 1 h. The top 1.0 ml of PBS was removed, sperm counts were performed and samples were diluted to a concentration of 10^6 spermatozoa/ml. The purified spermatozoa were used immediately for the isolation of RNA. Total RNA from somatic tissues and spermatozoa was extracted using GenElute Mammalian Total RNA Kit (Sigma-Aldrich, Italy) according to the manufacturer's instructions. RNA from each sample was quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and stored at –80 C until use.

Reverse transcription polymerase chain reaction and cDNA amplification

Reverse transcription polymerase chain reaction (RT-PCR) was carried out in a total volume of 20 μl of reaction mixture containing mRNA from pooled oocytes and total RNA from tissues and spermatozoa (1 μg total RNA), 1 μl of oligo$(dT)_{18}$ (0.5 μg/μl) and DEPC-treated water up to the final volume. RNA was denatured at 70 C for 5 min. Four μl of 5× reaction buffer, 1 μl of 10 mM dNTPs and 1 μl of RiboLock Ribonuclease inhibitor (20 U/ml) were added and the mixture was incubated at 37 C for 5 min. RT was performed with 1 μl (200 U) RevertAid H Minus M-MuLV RT for 1 hour at 42 C. Enzymes were inactivated at 70 C for 10 min. All reagents were purchased from M-Medical, Italy.

The correct mRNA retrotranscription was assessed by PCR. Primers for beta actin (EMBL DQ845171) and H2A histone family member Z (EMBL NM_174809) were designed based on the swine and bovine sequence respectively (Table 1), and the amplicons were used as positive controls in the following cDNA amplification.

Five primer pairs were designed based on the complete NLRP5 coding sequence (EMBL NM_001007814) and are reported in Table 1. All primer pairs were designed using the prediction program AmplifX 1.37. A negative control was also included in the analysis.

Polymerase chain reaction (PCR) was performed in a total volume of 15 μl of the following reaction mix: 0.6 μl 50 mM $MgCl_2$, 0.3 μl Taq DNA polymerase (5 U/μl), 1.5 μl 10 × PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1 μl 10 mM dNTPs and 6.25 pmol of each sequence-specific primer (Invitrogen, Italy). It was carried out in an Eppendorf thermal cycler (Eppendorf, Italy), using the following cycle: 94 C for 3 min, followed by 25 cycles of 94 C for 30 sec, 59–60 C for 30 sec and 72 C for 45 sec, with a final extension at 72 C for 5 min.

Genomic DNA extraction

Genomic DNA was extracted using a commercial kit (Quiagen, Italy). Two mg of porcine liver were frozen and ground in liquid nitrogen in a mortar. The ground tissue was suspended in 300 μl of cell lysis solution. Then, 1.5 μl of proteinase K (20 mg/ml) was

Table 2. Primer pairs used for PCR amplifications of each exon

Exon	Forward 5'- 3'	Reverse 5'- 3'	Amplicon size	Annealing temperature
1	acaggtcccattggcttcgtttac	tctgccccaatctcactcacttt	380 bp	58 C
2	gacacggtctcaactgttct	tcacacacatccgtttccac	159 bp	55 C
3	actctcttcacacccagtcccatt	aggatgagtgcattctggagctct	1917 bp	61 C
4	ggcagttgctcatgtcagaatcca	atgacacagccacacaaaggga	277 bp	58 C
5	tcacgttccctttttcccgtctgta	gtaaaagcagtgacgccctgaa	251 bp	59 C
6	ttgcctgtctctgcaggttggat	ggagtaccgctcacattagcttct	201 bp	58 C
7	tcttcaccgactgagctgtcatct	cactgctctgcactcgctgta	281 bp	60 C
8	tgtgtgccccgcagactaaac	aaccgtgtggtagacttgatctcc	228 bp	59 C
9	ccgcctatcgcagtttggtgaa	aagaccctaccgagtctcgt	191 bp	59 C
10	gggtctttcttggctgtgctga	accacaagtggtttggctggat	307 bp	59 C
11	tcccagagtaaggaagccccttt	tcgtctgctcaggagtcaca	467 bp	59 C

added, and the sample was incubated overnight at 55 C, and then for 30 min at 37 C with 1.5 μl of RNase A (4 mg/ml). The sample was cooled down to room temperature, and proteins were precipitated by adding 100 μl of protein precipitation solution and centrifuged at 13,000 × *g* for 3 min. The supernatant was poured into a clean tube containing 300 μl of 100% isopropanol, mixed and then centrifuged for 1 min at 13,000 × *g*. The tube was drained, and the pellet was washed by the addition of 300 μl of 70% ethanol and then centrifuged for 1 min at 13,000 × *g*. The pellet was air-dried at room temperature for 15 min, and DNA was rehydrated using 50 μl of DNA hydration solution and quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies). Finally, each sample was diluted to a final concentration of 5 ng/μl and stored at –20 C until use.

Genomic DNA amplification for RH mapping and sequencing

Primers for the complete sequence of each NLRP5 exon were designed based on the bovine gene sequence (Ensembl NP_001007815.1; Table 2) using the prediction program AmplifX 1.37.

PCR was performed in a total volume of 15 μl of the following reaction mix: 0.6 μl 50 mM $MgCl_2$, 0.3 μl Taq DNA polymerase (5 U/μl), 1.5 μl 10X PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1 μl 10 mM dNTPs and 6.25 pmol of each sequence-specific primer (Invitrogen, Italy). It was carried out in an Eppendorf thermal cycler (Eppendorf, Italy) using the following cycle: 94 C for 3 min, followed by 25–35 cycles of 94 C for 30 sec, 57–61 C for 30 sec and 72 C for 45 sec, with a final extension at 72 C for 5 min.

Sequencing of cDNA

PCR products were loaded onto a 2% agarose gel in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA) containing 0.5 μg/ml ethidium bromide and run at 80 V for 45 min. Fragments were visualized with a Molecular Imager ChemiDoc XRS System (Bio-Rad, Italy), and the image of each gel was recorded using the Quantity One 1-D Analysis Software (Bio-Rad, Italy). Amplicons for each exon of NLRP5 gene were excided from the agarose gel and eluted with a GenElute Gel Extraction Kit (Sigma-Aldrich, Italy). The amplicons were purified according to the manufacturer's instructions and quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies). PCR products were further purified with ExoSAP-IT (USB, Cleveland, OH, USA) and sequenced in both directions using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Italy). DNA sequencing by capillary electrophoresis was carried out with a ABI PRISM 3100 DNA Analyzer (Applied Biosystems).

Chromosomal mapping

The INRA porcine radiation hybrid (IMpRH) panel was used to map the NLRP5 gene. PCR reactions were performed as described above (annealing temperature, 57 C) using the following primers designed to amplify exon 6 and based on the sequence produced in the present study (EMBL AM748274): Fwd 5'-cggattaacgccagctcttgt-3' and Rev 5'-tagcttctgcagagtgcagtgtg-3'. PCR products of the expected size (157 bp) were visualized by agarose gel electrophoresis and ethidium bromide staining. Amplification was repeated twice. The marker was scored as positive (1) and negative (0) for each hybrid. The consensus data were uploaded onto the INRA RH database [32] and analyzed using the IMpRH web server (http://imprh.toulouse.inra.fr/).

Protein extraction, gel electrophoresis, western blot and immunostaining

Pig tissues (50 mg) were washed with PBS, and proteins were extracted following homogenization in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 10 g/l Nonidet-P40, 150 mM NaCl, 1 mM EDTA and protease inhibitor cocktails) at 4 C for 1 h. Proteins from the mouse ovary (50 mg), which was used as the positive control in cross-reactivity studies, were extracted following the same protocol. The supernatant was centrifuged at 10,000 × *g* for 3 min at 4 C. Protein concentration was assessed using a NanoDrop Spectrophotometer (NanoDrop Technologies). Fifty μg of proteins were separated by SDS-PAGE electrophoresis and then transferred onto a nitrocellulose membrane. The membrane was probed with a specific goat polyclonal antibody (diluted to 1:5,000) raised against a peptide of NLRP5 of mouse origin (NALP5 F-19, Santa Cruz Biotechnology, Germany). A specific donkey anti-goat IgG horseradish peroxidase conjugate (Santa Cruz Biotechnology) was used as the secondary antibody (1:2,000), and the presence of NLRP5 protein was visualized using an Immobilon Western HRP Kit (Millipore, Italy).

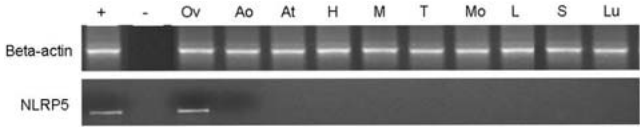

Fig. 1. Expression pattern of NLRP5 in porcine tissues assessed by PCR: ovary (Ov), aorta (Ao), adipose tissue (At), heart (H), muscle (M), tongue (T), medulla oblongata (Mo), liver (L), spleen (S), lung (Lu) and negative control (–). Porcine genomic DNA was used as the positive control for NLRP5 amplification (+). PCR for beta-actin amplicon was performed as the positive control for RT-PCR.

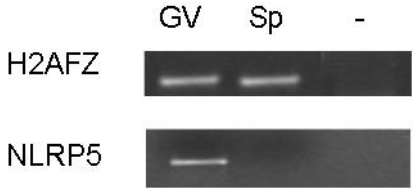

Fig. 2. Expression pattern of NLRP5 in germinal vesicle oocytes (GV) and spermatozoa (Sp). Negative control (–). Porcine genomic DNA was used as the positive control for NLRP5 amplification (+). Histone H2AFZ amplicon was used as the positive control for RT-PCR.

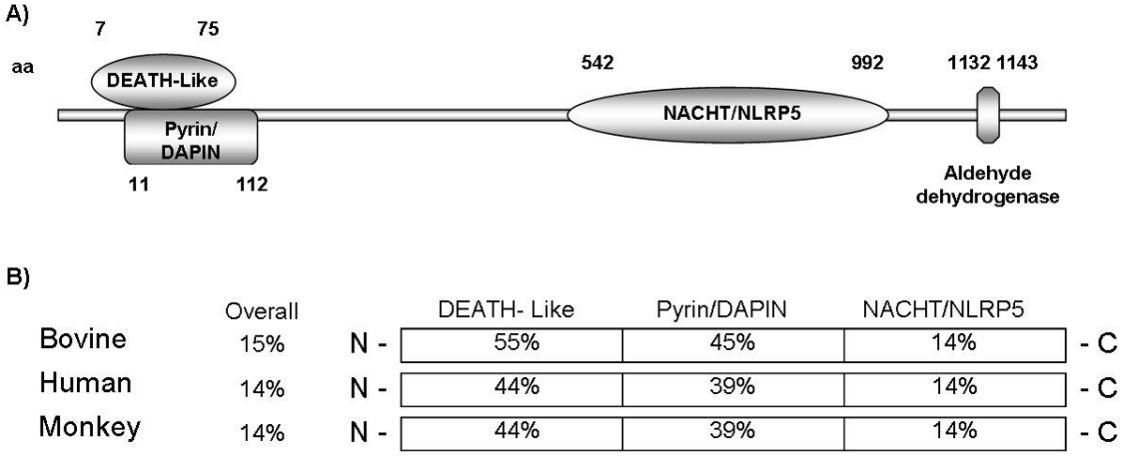

Fig. 3. (A) Porcine NLRP5 domains and motifs identified in the protein. (B) Amino acid identity of DEATH-Like, Pyrin/DAPIN and NACHT domains between porcine NLRP5 and bovine, human and monkey NLRP5. Overall amino acid identity is indicated on the left. The mouse protein was not aligned because it does not show two of the three domains considered.

Immunohistochemistry

The localization of the protein was investigated by immunohistochemistry. Ten-μm cryosections of ovary were air-dried, fixed in acetone for 3 min and stored at –20 C until use. After removing endogenous peroxydase activity in 1% H_2O_2 and blocking with donkey serum, ovarian sections were incubated with a specific goat polyclonal antibody (diluted to 1:100) raised against NLRP5 of mouse origin for 2 h at 37 C, and then incubated for 2 h at room temperature with the secondary biotinylated donkey anti-goat IgG antibody (Santa Cruz Biotechnology). Detection was carried out using a Vectastain ABC kit (Vector Labs, UK) and 3,3'-diaminobenzidine (DAB) as the substrate, according to the manufacturer's instructions. Sections were counterstained with hematoxylin and eosin. As the negative control, the specific antibody was omitted.

Results

Sequencing of cDNA and protein characterization

Primers were designed based on specific bovine exon sequences and on a bovine total cDNA sequence. The 3567 bp-long sequence produced in the present study and subsequent exon assembly was submitted to Genbank (EMBL AM941716).

The ClustalW multiple alignment program was used to align our sequence to the corresponding bovine (EMBL NM_001007814), human (EMBL AY154460), macaque monkey (EMBL NM_001127631) and murine (EMBL NM_001039143) protein-coding regions. To provide insight into amino acid homology among these sequences, the pig nucleotide sequence was converted into amino acid sequence data and aligned to the other mammalian NLRP5 protein sequences. The predicted porcine protein, 1188 amino acid residues, displays a similar length to the human (1200 aa) and macaque (1199 aa) proteins, and is longer than the bovine (1098 aa) and murine (1095 aa) proteins. A search within the InterPro database (http://www.ebi.ac.uk/InterProScan) was performed to investigate the protein structure. The predicted molecular mass is 130 kDa, and three major regions were identified, a DEATH-Like domain at the amino terminus (aa 7–75), a DAPIN or Pyrin domain (aa 11–112) and a NACHT nucleoside triphosphatase domain (aa 542–992). This global protein organization is typical of the NLRP5 protein. Furthermore, an aldehyde dehydrogenases cysteine active site (aa 1132–1143) was also found. This domain is

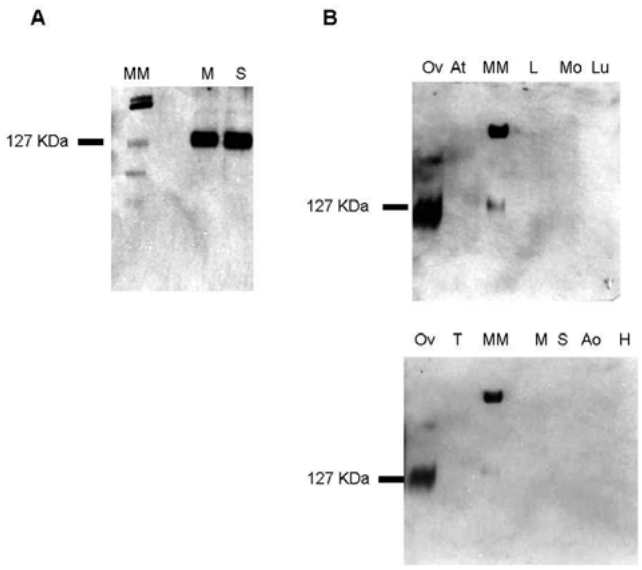

Fig. 4. Immunoblotting analysis. (A) Cross reactivity of NLRP5 antibody in mouse (M) and swine (S) ovarian cortex protein by western blot. Molecular mass (MM). (B) Distribution of NLRP5 protein in porcine tissues: adipose tissue (At), liver (L), medulla oblongata (Mo), lung (Lu), ovary (Ov), tongue (T), muscle (M), spleen (S), aorta (Ao) and heart (H). Molecular mass (MM).

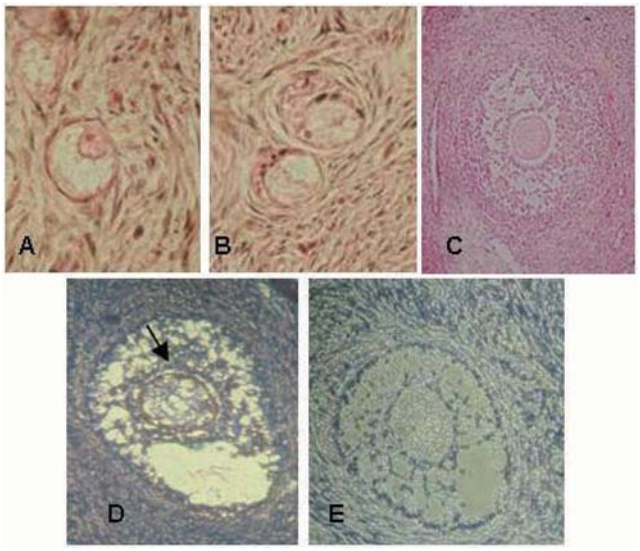

Fig. 5. Ovarian localization of porcine NLRP5 protein by immunohistochemistry. Ovarian sections showing primordial (A), primary (B), secondary (C) and antral follicles (D). Negative control for immunohistochemistry showing an antral follicle (E). The arrow indicates the protein localization in the subcortex of the oocyte. Original magnification 20×.

not present in the NLRP5 protein found in the bovine, mouse, human and non-human primates (Fig. 3A). The highest degree of homology was found in two functional domains, DEATH-Like and Pyrin/DAPIN. Moreover, it should be pointed out that the identities between amino acid sequences of identified domains are dramatically lower in NACHT/NLRP5 (Fig. 3B).

Chromosome mapping

The IMpRH panel was typed for the NLRP5 gene and the exon 6 specific amplicon was obtained in 32 of the 118 hybrid cell lines (retention frequency of 0.19). In accordance with the work by Ma *et al*. [33], the mapping results assigned NLRP5 to SSC6. The closest marker, in the present study was SW193 in a region of conserved synteny with bovine chromosome 18, human chromosome 19 and mouse chromosome 7.

Tissue and germ cell expression of NLRP5

Semi-quantitative analysis of the expression patterns of NLRP5 in somatic tissues (heart, liver, lung, spleen, medulla oblongata, tongue, muscle, adipose and aorta) and germ line tissue (ovary) was carried out. Beta-actin was used as the internal control to standardize expression levels for reverse-transcription, as it is known to be expressed in all tissues; Histone H2AFZ was used as the positive control for germ cells. A negative control was also included in the analysis. The presence of PCR products showed NLRP5 to be strongly expressed in ovaries, but expression was not detected in the other tissues (Fig. 1). Further transcription analysis showed NLRP5 to be specifically expressed in germinal vesicle oocytes but not in spermatozoa (Fig. 2).

NLRP5 protein localization

NLRP5 protein expression was assessed by western blot using a goat polyclonal antibody raised against NLRP5 of mouse origin as the primary antibody. The cross-reactivity of the antibody with porcine tissues was validated by using mouse ovarian extracts as positive controls (Fig. 4A). After this first preliminary step, NLRP5 protein expression was investigated in all the tissues sampled, and the results showed that the protein was expressed in the ovary alone (Fig. 4B). This result is consistent with the transcription data produced in this study and in previous studies in other species [25]. The molecular mass of the porcine protein, 130 KDa, corresponds to that of the mouse, which was used as the positive control, and is in the range of all NLRP5 proteins characterized so far in other species [24, 34]

To further localize the protein within the ovarian tissue, cryosections of pig ovaries were incubated with the same antibody specific for NLRP5 peptide used in the western blot analysis. The protein was found to be present with a similar labelling intensity only in the subcortex of oocytes in antral follicles (Fig. 5D–E). The protein was not detected in oocytes from primordial, primary or secondary follicles (Fig. 5A–C). Follicles larger than the antral follicles were not observed in this study.

Discussion

Gametes are transcriptionally quiescent at the time of fertilization, and activation of the embryonic genome does not occur until the late one-cell to early 2-cell stage of preimplantation development in mice [35], 4-cell stage in pigs [36, 37], 4–8-cell stage in

humans [28, 38] and non-human primates [39] and 8–16-cell stage in cattle [40]. Processing of the sperm nucleus, organization of the two pronuclei and the first cell divisions, as well as activation of the embryonic genome are regulated by pre-existing factors in the egg cytoplasm encoded by maternal genes [19]. The NLRP5 gene has one of the earliest effects on embryogenesis; fertilized oocytes from NLRP5 knock-out mice do not progress beyond the 2-cell stage [15]. NLRP5 transcripts accumulate during oogenesis and are translated into protein. During meiotic maturation and ovulation, NLRP5 transcripts are degraded, but the cytoplasmic protein persists until the early blastocyst stage, suggesting a physiological role beyond the first embryonic cleavage.

In this study, we assessed the expression patterns of pig NLRP5 in somatic tissues, oocytes and spermatozoa. NLRP5 could not be detected in any tissue except the ovary; the transcript was also present in germinal vescicle oocytes, but not in spermatozoa. The tissue distribution of NLRP5 expression supports a role in reproductive function as in mice and cattle. Indeed, NLRP5 and Zar1 gene are the first germ cell-specific maternal effect genes identified in pig so far [41].

The 3.57-kb ORF of the pig NLRP5 gene here assessed has a size similar to those of mouse (3.46-kb) [23], bovine (3.54-kb) [42] human (3.88 kb) [27] and macaque (3.88 kb) [26] transcripts. The primers used in the present study were designed based on the bovine sequence (Ensembl ENSBTAG00000013247), and all 11 regions corresponding to bovine exons were satisfactorily amplified. The different length of the pig coding sequence, though, suggests that a different gene organization could exist. Indeed, human and mouse NLRP5 genes have similar ORF sizes resulting from different number of exons; the human gene has 15 exons (Ensembl ENSG00000171487), and the mouse gene 16 exons (Ensembl ENSMUSG00000015721).

The pig ORF encodes for a putative protein of 1188 aa with a predicted molecular mass of 130 kDa. This protein is longer than the bovine (1098 aa) and mouse (1111 aa) proteins, and shorter than the human (1200 aa) and macaque (1199 aa) proteins. Structural analysis of the NLRP5 protein in different species showed a different distribution and the presence of different domains. Two domains characteristic of the NLRP family [31] are conserved in the pig, bovine, human and macaque monkeys protein, but the mouse protein has only the NACHT domain, as the authors of the present paper ascertained by running *in silico* analyses in the InterPro database. The N-terminal Pyrin/DAPIN domain, identified as a putative protein-protein interaction domain, is thought to function in the apoptotic and inflammatory signaling pathway. This domain is a member of the six-helix bundle death domain-fold superfamily [43–46] and is followed by a NACHT domain, a predicted nucleoside triphosphatase (NTPase) domain, which is found in animal, fungal and bacterial apoptotic proteins [47]. These two domains are often associated with a DEATH-Like domain, a homotypic protein interaction module related to the Death Effector Domain (DED) and the Caspase Recruitment Domain (CARD) [48]. Furthermore, an aldehyde dehydrogenase cysteine active site was identified for the first time in the present study, as it has not been previously identified in the NLRP5 proteins of other species. Aldehyde dehydrogenases (ALDHs) are a group of enzymes that catalyse the oxidation of aldehydes and are largely conserved throughout the different classes of the enzyme. If supported with a validated ALDH enzyme activity of pig NLRP5, this finding may suggest a possible role for this protein in detoxification of aldehydes as previously shown in preimplantation mouse embryos [50].

In the present study, the pig NLRP5 gene was assigned to chromosome SSC6q11–21 by RH mapping, the closest marker being SW193. Recently, Ma *et al*. [33] assigned the NLRP5 gene to the long arm of chromosome 6 (SSC6q21–22) linked to marker CL388309. To investigate the discrepancy in map position, the pairs of primers designed and used in the two studies were aligned to the bovine genomic sequence (Ensembl ENSBTAG00000013247). The primers used by Ma *et al*. correspond to bovine exon 3, whereas the primers used in the present study amplify exon 6. Unfortunately, no pig genomic contig sequence including the SW193 and CL388309 markers and the fragments amplified in both studies is available. Therefore, the actual distance between the two regions cannot be precisely assessed. Furthermore, partial alignments of the NLRP5 sequence on unfinished genomic contigs revealed differences in intron length, with the pig intron sequence being longer than that of the bovine. Previous studies have identified in this chromosome 6 region, QTLs for "total newborn" [51], "newborn alive" and "mummified piglet" [52] traits. In humans, the corresponding region in chromosome 19 is known to carry six NLRP genes (NLRP 9, NLRP 11, NLRP 4, NLRP 13, NLRP 8 and NLRP 5). Finally, the syntenic region in bovine chromosome 18 contains a QTL region for stillbirth (maternal effect) and nonreturn rate at 90 days (maternal and paternal effect), as well as dystocia (direct effect) [53].

In the present study, the tissue distribution of the NLRP5 protein was assessed by western blot. To further characterize the localization of this protein, immunohistochemical staining was performed, which showed that NLRP5 is a cytoplasmatic protein present in the ovaries, specifically in the subcortex of oocytes of antral follicles, which has also been recently shown in mice by Li *et al*. [54]. We showed that the expression of this gene is restricted to gonad cells and female germ cells, confirming data previously reported in other species [1, 3, 15, 27, 42]. Moreover, the protein identified in the present study has a molecular mass (130 kDa) in the range of the NLRP5 proteins that have been sequenced in other species [24, 27, 29].

The genomic sequence, the expression in female gonads and germ cells and assignment to a QTL region affecting reproductive traits in SSC6 confirmed NLRP5 as a candidate gene for fertility traits related to gametogenesis, oogenesis and preimplantation development.

Although little is known about maternal effects in pigs, increasing evidence in different mammalian species indicates that maternal factors present in oocytes perform a critical role in biological events following fertilization [13, 20, 55, 56]. Biological evidence for impact on early embryonic development has been obtained using NLRP5-null female mice, which show a block in embryonic development [15]. The spatial expression shown in the present study suggests the functional relevance for maternal effects on female gametes in pigs.

Further characterization of the oocyte-selective NLRPs in multiple species along with signaling pathway/interactive protein analysis may eventually unravel an oocyte-specific genetic network crucial for preimplantation development of embryos.

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References

1. **Dade S, Callebaut I, Paillisson A, Bontoux M, Dalbies-Tran R, Monget P**. *In silico* identification and structural features of six new genes similar to MATER specifically expressed in the oocyte. *Biochem Biophys Res Commun* 2004; 324: 547–553.
2. **Goto T, Jones GM, Lolatgis N, Pera MF, Trounson AO, Monk M**. Identification and characterisation of known and novel transcripts expressed during the final stages of human oocyte maturation. *Mol Reprod Dev* 2002; 62: 13–28.
3. **Hamatani T, Carter MG, Sharov AA, Ko MS**. Dynamics of global gene expression changes during mouse preimplantation development. *Dev Cell* 2004; 6: 117–131.
4. **Rajkovic A, Yan MSC, Klysik M, Matzuk M**. Discovery of germ cell-specific transcripts by expressed sequence tag database analysis. *Fertil Steril* 2001; 76: 550–554.
5. **Zeng F, Schultz RM**. Gene expression in mouse oocytes and preimplantation embryos: use of suppression subtractive hybridization to identify oocyte- and embryo-specific genes. *Biol Reprod* 2003; 68: 31–39.
6. **Kempisty B, Antosik P, Bukowska D, Jackowska M, Lianeri M, Jaskowski JM, Jagodzinski PP**. Analysis of selected transcript levels in porcine spermatozoa, oocytes, zygotes and two-cell stage embryos. *Reprod Fertil Dev* 2008; 20: 513–518.
7. **Boerke A, Dieleman SJ, Gadella BM**. A possible role for sperm RNA in early embryo development. *Theriogenology* 2007; 68 (Suppl): S147–S155.
8. **Bebbere D, Bogliolo L, Ariu F, Fois S, Leoni GG, Tore S, Succu S, Berlinguer F, Naitana S, Ledda S**. Expression pattern of zygote arrest 1 (ZAR1), maternal antigen that embryo requires (MATER), growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) genes in ovine oocytes and *in vitro*-produced preimplantation embryos. *Reprod Fertil Dev* 2008; 20: 908–915.
9. **Wu X, Viveiros MM, Eppig JJ, Bai Y, Fitzpatrick SL, Matzuk MM**. Zygote arrest 1 (Zar1) is a novel maternal-effect gene critical for the oocyte-to-embryo transition. *Nat Genet* 2003; 33: 187–191.
10. **Bortvin A, Goodheart M, Liao M, Page DC**. Dppa3 / Pgc7 / stella is a maternal factor and is not required for germ cell specification in mice. *BMC Dev Biol* 2004; 4: 2.
11. **Payer B, Saitou M, Barton SC, Thresher R, Dixon JP, Zahn D, Colledge WH, Carlton MB, Nakano T, Surani MA**. Stella is a maternal effect gene required for normal early development in mice. *Curr Biol* 2003; 13: 2110–2117.
12. **Burns KH, Viveiros MM, Ren Y, Wang P, DeMayo FJ, Frail DE, Eppig JJ, Matzuk MM**. Roles of NPM2 in chromatin and nucleolar organization in oocytes and embryos. *Science* 2003; 300: 633–636.
13. **Dean J**. Oocyte-specific genes regulate follicle formation, fertility and early mouse development. *J Reprod Immunol* 2002; 53: 171–180.
14. **Varani S, Matzuk MM**. Phenotypic effects of knockout of oocyte-specific genes. *Ernst Schering Res Found Workshop* 2002; 41: 63–79.
15. **Tong ZB, Gold L, Pfeifer KE, Dorward H, Lee E, Bondy CA, Dean J, Nelson LM**. Mater, a maternal effect gene required for early embryonic development in mice. *Nat Genet* 2000; 26: 267–268.
16. **Meirelles FV, Caetano AR, Watanabe YF, Ripamonte P, Carambula SF, Merighe GK, Garcia SM**. Genome activation and developmental block in bovine embryos. *Anim Reprod Sci* 2004; 82–83: 13–20.
17. **Moor R, Dai Y**. Maturation of pig oocytes *in vivo* and *in vitro*. *Reprod Suppl* 2001; 58: 91–104.
18. **Dieleman SJ, Hendriksen PJ, Viuff D, Thomsen PD, Hyttel P, Knijn HM, Wrenzycki C, Kruip TA, Niemann H, Gadella BM, Bevers MM, Vos PL**. Effects of *in vivo* prematuration and *in vivo* final maturation on developmental capacity and quality of preimplantation embryos. *Theriogenology* 2002; 57: 5–20.
19. **Evsikov AV, Marin de Evsikova C**. Gene expression during the oocyte-to-embryo transition in mammals. *Mol Reprod Dev* 2009; 76: 805–818.
20. **Donnison M, Pfeffer PL**. Isolation of genes associated with developmentally competent bovine oocytes and quantitation of their levels during development. *Biol Reprod* 2004; 71: 1813–1821.
21. **Hwang KC, Lee HY, Cui XS, Kim JH, Kim NH**. Identification of maternal mRNAs in porcine parthenotes at the 2-cell stage: a comparison with the blastocyst stage. *Mol Reprod Dev* 2005; 70: 314–323.
22. **Telford NA, Watson AJ, Schultz GA**. Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Mol Reprod Dev* 1990; 26: 90–100.
23. **Tong ZB, Nelson LM**. A mouse gene encoding an oocyte antigen associated with autoimmune premature ovarian failure. *Endocrinology* 1999; 140: 3720–3726.
24. **Pennetier S, Perreau C, Uzbekova S, Thelie A, Delaleu B, Mermillod P, Dalbies-Tran R**. MATER protein expression and intracellular localization throughout folliculogenesis and preimplantation embryo development in the bovine. *BMC Dev Biol* 2006; 6: 26.
25. **Ponsuksili S, Brunner RM, Goldammer T, Kuhn C, Walz C, Chomdej S, Tesfaye D, Schellander K, Wimmers K, Schwerin M**. Bovine NALP5, NALP8, and NALP9 genes: assignment to a QTL region and the expression in adult tissues, oocytes, and preimplantation embryos. *Biol Reprod* 2006; 74: 577–584.
26. **McDaniel P, Wu X**. Identification of oocyte-selective NLRP genes in rhesus macaque monkeys (*Macaca mulatta*). *Mol Reprod Dev* 2009; 76: 151–159.
27. **Tong ZB, Bondy CA, Zhou J, Nelson LM**. A human homologue of mouse Mater, a maternal effect gene essential for early embryonic development. *Hum Reprod* 2002; 17: 903–911.
28. **Hamatani T, Ko M, Yamada M, Kuji N, Mizusawa Y, Shoji M, Hada T, Asada H, Maruyama T, Yoshimura Y**. Global gene expression profiling of preimplantation embryos. *Hum Cell* 2006; 19: 98–117.
29. **Tong ZB, Gold L, De Pol A, Vanevski K, Dorward H, Sena P, Palumbo C, Bondy CA, Nelson LM**. Developmental expression and subcellular localization of mouse MATER, an oocyte-specific protein essential for early development. *Endocrinology* 2004; 145: 1427–1434.
30. **Ting JP, Davis BK**. CATERPILLER: A novel gene family important in immunity, cell death, and diseases. *Annu Rev Immunol* 2005; 23: 387–414.
31. **Tschopp J, Martinon F, Burns K**. NALPs: a novel protein family involved in inflammation. *Nat Rev Mol Cell Biol* 2003; 4: 95–104.
32. **Milan D, Hawken R, Cabau C, Leroux S, Genet C, Lahbib Y, Tosser G, Robic A, Hatey F, Alexander L, Beattie C, Schook L, Yerle M, Gellin J**. IMpRH server: an RH mapping server available on the Web. *Bioinformatics* 2000; 16: 558–559.
33. **Ma J, Milan D, Rocha D**. Chromosomal assignment of the porcine NALP5 gene, a candidate gene for female reproductive traits. *Anim Reprod Sci* 2009; 112: 397–401.
34. **Tong ZB, Nelson LM, Dean J**. Mater encodes a maternal protein in mice with a leucine-rich repeat domain homologous to porcine ribonuclease inhibitor. *Mamm Genome* 2000; 11: 281–287.
35. **Flach G, Johnson MH, Braude PR, Taylor RA, Bolton VN**. The transition from maternal to embryonic control in the 2-cell mouse embryo. *Embo J* 1982; 1: 681–686.
36. **Freitag M, Dopke HH, Niemann H, Elsaesser F**. Ontogeny of RNA synthesis in preimplantation pig embryos: an ultrastructural and autoradiographic analysis. *J Reprod Fertil Abstr Ser* 1988; 1: 11.
37. **Tomanek M, Kopecny V, Kanka J**. Genome reactivation in developing early pig embryos: an ultrastructural and autoradiographic analysis. *Anat Embryol (Berl)* 1989; 180: 309–316.
38. **Braude P, Bolton V, Moore S**, Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 1988; 332: 459–461.
39. **Zheng P, Patel B, McMenamin M, Paprocki AM, Schramm RD, Nagl NGJ, Wilsker D, Wang X, Moran E, Latham KE**. Expression of genes encoding chromatin regulatory factors in developing rhesus monkey oocytes and preimplantation stage embryos: possible roles in genome activation. *Biol Reprod* 2004; 70: 1419–1427.
40. **Memili E, First NL**. Control of gene expression at the onset of bovine embryonic development. *Biol Reprod* 1999; 61: 1198–1207.
41. **Uzbekova S, Roy-Sabau M, Dalbies-Tran R, Perreau C, Papillier P, Mompart F, Thelie A, Pennetier S, Cognie J, Cadoret V, Royere D, Monget P, Mermillod P**. Zygote arrest 1 gene in pig, cattle and human: evidence of different transcript variants in male and female germ cells. *Reprod Biol Endocrinol* 2006; 4: 12.
42. **Pennetier S, Uzbekova S, Perreau C, Papillier P, Mermillod P, Dalbies-Tran R**. Spatio-temporal expression of the germ cell marker genes MATER, ZAR1, GDF9, BMP15, and VASA in adult bovine tissues, oocytes, and preimplantation embryos. *Biol Reprod* 2004; 71: 1359–1366.
43. **Bertin J, DiStefano PS**. The PYRIN domain: a novel motif found in apoptosis and inflammation proteins. *Cell Death Differ* 2000; 7: 1273–1274.
44. **Fairbrother WJ, Gordon NC, Humke EW, O'Rourke KM, Starovasnik MA, Yin JP, Dixit VM**. The PYRIN domain: a member of the death domain-fold superfamily. *Protein Sci* 2001; 10: 1911–1918.
45. **Martinon F, Hofmann K, Tschopp J**. The pyrin domain: a possible member of the death domain-fold family implicated in apoptosis and inflammation. *Curr Biol* 2001;

11: R118–120.

46. **Staub E, Dahl E, Rosenthal A**. The DAPIN family: a novel domain links apoptotic and interferon response proteins. *Trends Biochem Sci* 2001; 26: 83–85.
47. **Koonin EV, Aravind L**. The NACHT family—a new group of predicted NTPases implicated in apoptosis and MHC transcription activation. *Trends Biochem Sci* 2000; 25: 223–224.
48. **Feinstein E, Kimchi A, Wallach D, Boldin M, Varfolomeev E**. The death domain: a module shared by proteins with diverse cellular functions. *Trends Biochem Sci* 1995; 20: 342–344.
49. **Tu GC, Weiner H**. Evidence for two distinct active sites on aldehyde dehydrogenase. *J Biol Chem* 1988; 263: 1218–1222.
50. **Rout UK, Armant DR**. Expression of genes for alcohol and aldehyde metabolizing enzymes in mouse oocytes and preimplantation embryos. *Reprod Toxicol* 2002; 16: 253–258.
51. **Cao H, Robinson JA, Jiang Z, Melville JS, Golovan SP, Jones MW, Verrinder Gibbins AM**. A high-resolution radiation hybrid map of porcine chromosome 6. *Anim Genet* 2004; 35: 367–378.
52. **Holl JW, Cassady JP, Pomp D, Johnson RK**. A genome scan for quantitative trait loci and imprinted regions affecting reproduction in pigs. *J Anim Sci* 2004; 82: 3421–3249.
53. **Kuhn C, Bennewitz J, Reinsch N, Xu N, Thomsen H, Looft C, Brockmann GA, Schwerin M, Weimann C, Hiendleder S, Erhardt G, Medjugorac I, Forster M, Brenig B, Reinhardt F, Reents R, Russ I, Averdunk G, Blumel J, Kalm E**. Quantitative trait loci mapping of functional traits in the German Holstein cattle population. *J Dairy Sci* 2003; 86: 360–368.
54. **Li L, Baibakov B, Dean J**. A subcortical maternal complex essential for preimplantation mouse embryogenesis. *Dev Cell* 2008; 15: 416–425.
55. **Barritt J, Willadsen S, Brenner C, Cohen J**. Cytoplasmic transfer in assisted reproduction. *Hum Reprod Update* 2001; 7: 428–435.
56. **Ohsugi M, Zheng P, Baibakov B, Li L, Dean J**. Maternally derived FILIA-MATER complex localizes asymmetrically in cleavage-stage mouse embryos. *Development* 2008; 135: 259–269.