Molecular Human Reproduction, Vol.15, No.8 pp. 499–506, 2009
Advanced Access publication on June 20, 2009 doi:10.1093/molehr/gap048

MHR Basic science of reproductive medicine

ORIGINAL RESEARCH

MATER protein as substrate of PKCε in human cumulus cells

T. Maraldi[1,4], M. Riccio[1], P. Sena[1], L. Marzona[1], A. Nicoli[2], A. La Marca[3], S. Marmiroli[1], J. Bertacchini[1], G. La Sala[2], and A. De Pol[1]

[1]Department of Anatomy and Histology, University of Modena and Reggio Emilia, Via Del Pozzo 71, 41100 Modena, Italy [2]Department of Obstetrics and Gynecology, Arcispedale Santa Maria Nuova, V.le Risorgimento 80, 42100 Reggio Emilia, Italy [3]Mother-Infant Department, Unit of Obstetrics and Gynecology, University of Modena and Reggio Emilia, Via Del Pozzo 71, 41100 Modena, Italy

[4]Correspondence address. Fax: +39-059-4224861; E-mail: tullia.maraldi@unimo.it

ABSTRACT: High activity of the phosphoinositide 3-kinase/Akt pathway in cumulus cells plays an important role in FSH regulation of cell function and Protein Kinase C epsilon (PKCε) collaborates with these signalling pathways to regulate cell proliferation. Relevant roles in follicular development are played by Maternal Antigen That Embryos Require (MATER) that is a cumulus cell- and oocyte-specific protein dependent on the maternal genome. We recently demonstrated that human MATER localizes at specific domains of oocytes and, for the first time, also in cumulus cells. MATER contains a carboxy-terminal leucine-rich repeat domain involved in protein–protein interactions regulating different cellular functions. Here we investigated the functional role of MATER. Thus, we performed coimmunoprecipitation experiments using HEK293T cells expressing human MATER; a similar approach was then followed in human cumulus/follicular cells. In *MATER*$^{+}$HEK293T cells, we observed that this protein acts as a phosphorylation substrate of PKCε. Western blot experiments indicate that, unlike oocytes, human cumulus cells express PKCε. Immunoprecipitation and confocal analysis suggest for the first time that MATER protein interacts with this protein kinase in cumulus cells under physiological conditions. Since PKCε is known to collaborate with antiapoptotic signalling pathways, this suggests a novel mechanism for the function of MATER in follicular maturation.

Key words: cumulus cells / follicular development / human ovary / MATER / PKC

Introduction

Over the past few years a number of maternal effect genes, specifically expressed in the gonads, have been studied to understand the molecular mechanisms governing oocyte and early embryo development. Maternal effect genes encode for proteins and transcripts that accumulate during oogenesis and are necessary to support the development of preimplantation embryo (Latham, 1999).

Among these, two growth factors namely growth differentiation factor 9 and bone morphogenetic protein 15, are essential for normal follicular development (Laitinen *et al.*, 1998; Galloway *et al.*, 2000; Gilchrist *et al.*, 2004). The pivotal role of these factors in folliculogenesis and oocyte development is revealed by the discovery of their involvement in relaying communication between the oocyte and its surrounding somatic cells. Interactions between the developing gametes and neighbouring somatic cells are crucial for fertility because this complex dialogue is essential for the coordinated development of both germ cell and somatic cells (Eppig, 2001; Cecconi *et al.*, 2004).

Another maternal effect gene is Maternal Antigen that Embryos Require (MATER), supposed to be an oocyte specific protein. MATER (Nlrp5—Mouse Genome Informatics) was one of the earliest maternal effect genes molecularly characterized in mice (Tong *et al.*, 2000a). The 125 kDa protein, encoded by a single-copy gene on Chromosome 7 (Tong *et al.*, 2000b), has been more recently identified as a NALP protein (Tschopp *et al.*, 2003), the largest clade in the Caterpillar family (Harton *et al.*, 2002). NALP (NACHT-, leucine rich repeat (LRR) and pyrin domain (PYD)-containing) proteins are characterized by a modular structure, with an N-terminal-based NACHT (NAIP; CIIA; HET-E; and TP1) domain with NTPase function which seems to mediate heterotypic interaction (Damiano *et al.*, 2004). NALP proteins contain an amino-terminal PYD and carboxy-terminal LRR region. PYD domains are present in the members of the death superfamily (Martinon *et al.*, 2001; Pawlowski *et al.*, 2001). Proteins carrying a PYD are mainly involved in inflammation, apoptosis and NF-kB transcription factor signalling (Koonin and Aravind, 2000; Hoffman *et al.*, 2001; Gumucio *et al.*, 2002). Moreover, MATER, also called NALP5, contains the LRR domain, known to be involved in protein–protein interactions that regulate different cellular functions (Lee and Vallee, 2006). The superfamily of LRR proteins can be subdivided into six subfamilies (Kajava, 1998). According to this classification, functional studies based on knock-out mouse models

demonstrated that *Mater*-null females are fertilized normally, but the resulting embryos arrested the development at the 2-cell stage (Tong *et al*., 2000b). *Mater*-null ovaries had a normal complement of all stages of developing follicles, corpora lutea were also present and the number of ovulated ova was similar to those from normal females (Tong *et al*., 2004).

Our previous work described that the distribution of mouse MATER is regulated in a manner typical of maternal effect genes: *Mater* is transcripted only during oogenesis, and the protein is stable until the morula stage and early blastocyst but disappears in late blastocyst (Tong *et al*., 2004).

Our recent article showed for the first time that during human folliculogenesis the simultaneous detection of MATER in follicular cells and oocytes starts in late primary follicles, when the maturation process is activated, persists for the entire process and also shows high levels after ovulation. MATER is expressed in cumulus oophorus cells, in intimate connection with the oocyte, but not in mural granulosa cells, spatially separated from the oocyte (Sena *et al*., 2009). Bidirectional signalling between the oocyte and the surrounding somatic cells is crucial for coordinated development of both germ and somatic cell compartments (Eppig *et al*., 1997, 2001; Hutt and Albertini, 2007).

Li *et al*. recently identified a subcortical maternal complex (SCMC) that assembles during oocyte growth and is essential for zygotes to progress beyond the first embryonic cell divisions. At least four maternally encoded proteins contribute to this complex: FLOPED, MATER and TLE6 interact with each other, although FILIA binds independently to MATER. The SCMC, located in the subcortex of oocytes, is excluded from regions of cell–cell contact in the cleavage-stage embryo and segregates to the outer cells of the morula and blastocyst. *Floped*$^{tm/tm}$ and/or *Mater*$^{tm/tm}$ oocytes lack the SCMC but can be fertilized. However, these embryos do not progress beyond cleavage stage development and female mice are sterile (Li *et al*., 2008).

The aim of this study was to obtain insights on MATER function during human folliculogenesis. We demonstrated that MATER is a physiological substrate of Protein Kinase C epsilon (PKCε) in human cumulus cells. PKCε, a member of the novel PKC group, is believed to function as an antiapoptotic protein, so it is possible that MATER–PKCε interaction collaborates with other signalling pathways, such as phosphoinositide (PI) 3-K and Akt, to regulate cell survival and cell death. This new step in the characterization of MATER binding proteins suggests a possible mechanism for the involvement of MATER in the promotion of follicular maturation.

Materials and Methods

Human cumulus cells and oocytes collection

Isolated oocytes and follicular cells were collected by pick-up technique during clinical *in vitro* fertilization procedures. These samples were collected by the U.O. Ginecologia e Ostetricia (Arcispedale S. Maria Nuova Reggio Emilia) with informed consent according to Italian law and ethical committee guideline.

Cell culture and transfections

Human embryonic kidney (HEK) epithelial 293T cells were grown in Dulbecco's Modified Eagle's Medium High Glucose (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% Foetal Calf Serum (EuroClone Ltd, Wetherby, Yorkshire, UK).

Transient transfections of 293T cells were performed by the calcium-phosphate method (Cenni *et al*., 2003).

Plasmids

The human MATER expression vector (pcDNA/His/HuMater-26ORF) was kindly provided by Dr Tong Z.B (NIH) and was constructed by subcloning a BamHI–ApaI fragment of human *MATER* cDNA (encoding human MATER residue 25–1199) into BamHI–ApaI sites of pcDNA3.1/HisB vector (Invitrogen, Carlsbad, CA, USA).

The mouse cDNA of PKCε with a BglII site in the 5′ and 3′ termini was obtained by PCR (sense primer 5′-GGAAGATCTTCCATGGTAGTG TTCAATGGCCTTC-3′, antisense primer 5′-GGAAGATCTTCCTCA GGGCTACAGGTCTTCSCC-3′) and subcloned into the BglII site of the pEGFPC1 eukaryotic expression vector, in frame with the green fluorescent protein (GFP)-tag.

Preparation of cell extracts

Subconfluent cells were extracted by addition of RIPA buffer (20 mM Tris-Cl, pH 7.0, 1% NP-40, 150 mM NaCl, 10% Glycerol, 10 mM EDTA, 20 mM NaF, 5 mM Sodium Pyrophosphate, 1 mM Na_3VO_4, and freshly added Sigma-Aldrich Protease Inhibitor Cocktail) at 4°C for 10 min. Lysates were cleared by centrifugation and used for immunoprecipitation experiments, as described below. A total of 50 μl of the total lysate was immediately boiled in SDS sample buffer, resolved, and immunoblotted with the indicated antibodies. The following antibodies were used: anti-PKCε from Sigma-Aldrich (St. Louis, MO, USA); anti-actin, anti-NALP5 (MATER), anti-Akt, from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-pPDK1, anti-Akt phosphoserine 473 (pAkt S473), anti-PKC phosphosubstrate and anti-Akt phosphosubstrate, from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Immunoprecipitation and electrophoresis

Equal amounts of precleared lysates, whose protein concentration was determined by the Bradford method, were incubated overnight with anti-Nalp5 (Santa Cruz Biotech., Santa Cruz, CA, USA), anti-PKCε (Sigma-Aldrich, St. Louis, MO, USA) (3 μg all). Then the two samples were treated with 30 μl of anti-goat IgG-Agarose (Sigma-Aldrich, St. Louis, MO, USA) and 50% (v/v) of protein A/G agarose slurry (Santa Cruz Biotech, Santa Cruz, CA, USA) respectively at 4°C with gentle rocking for 1 h. Pellets were washed twice with PBS plus 1% NP-40, twice in TNE (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM EDTA), once with 10 mM Tris-Cl, pH 7.4, boiled in Laemmli sample buffer, and centrifuged. Supernatants were loaded onto SDS-polyacrylamide gel, blotted on Immobilon-P membranes (Millipore, Billerica, MA, USA), and processed by western blot with the indicated antibody, detected by Supersignal substrate chemiluminescence detection kit (Pierce, Rockford, IL, USA). Quantification of the signal was obtained by chemiluminescence detection on a Kodak Image Station 440CF and analysis with the Kodak 1D Image software.

PKC kinase assay and phosphosubstrate analysis

All proteins (MATER expressed in 293T and PKCε wild type or PKCε/GFP overexpressed in 293T) were immunoprecipitated as described above. PKC Kinase *in vitro* Assay were performed adding PKCε samples to MATER immunoprecipitated in a buffer comprising 20 mM Tris-Cl, pH 7.5, 25 mM $MgCl_2$, 1 mM DTT, 2 mM Na_3VO_4, 80 mM ATP and carried out for 15 min at 37°C. Pellets were then immediately boiled in SDS sample buffer and immunoblotted with the indicated antibodies.

MATER bands were localized among the proteins recognized by Phospho-(Ser) PKC substrate antibody (Cell Signaling Technology Inc., Beverly, MA, USA).

Immunofluorescence confocal microscopy

Isolated oocytes and cumulus cells were fixed *in toto* in 4% paraformaldehyde in PBS and permeabilized with 1% Triton X-100 in PBS for 10 min.

After a treatment with 3% BSA in PBS for 30 min at room temperature, the oocytes and cumulus cells were incubated with the primary antibodies diluted in PBS containing 3% BSA [goat anti-NALP5 diluted 1:20, rabbit anti-PKCε diluted 1:50 and mouse anti-phosphatidylinositol (3,4,5)-triphosphate (PIP_3) (Echelon, Salt Lake City, UT, USA) diluted 1:50 overnight at 4°C]. After washing in PBS containing 3% BSA, the samples were incubated 1 h at room temperature with the secondary Abs diluted 1:200 in PBS containing 3% BSA (bovine anti-goat FITC, sheep anti-rabbit Cy3 and Sheep anti-Mouse Cy3). After washing in PBS the samples were then mounted with anti-fading medium (0.21 M DABCO and 90% glycerol in 0.02 M Tris, pH 8.0). Negative controls consisted of samples not incubated with the primary antibody. To confirm antibody specificity a control was performed pre-incubating anti-Nalp5 antibody with an excess of the blocking peptide (20 μg of peptide and 2 μg of antibody) for 1 h at 37°C. The multi-labelling immunofluorescence experiments were carried out avoiding cross-reactions between primary and secondary antibodies.

The confocal imaging was performed on a Leica TCS SP2 AOBS confocal laser scanning microscope. For FITC and Cy3 double detection, the samples were sequentially excited with the 488 nm lines of the argon laser and the 543 nm line of a HeNe laser. The excitation and the detection of the samples were carried out in sequential mode to avoid overlapping of the two signals. Optical sections were obtained at increments of 0.3 μm in the *z*-axis and were digitized with a scanning mode format of 512 × 512 pixels and 256 gray levels. The confocal serial sections were processed with the Leica LCS software to obtain three-dimensional projections. The image rendering was performed by adobe Photoshop software. Co-localization was evaluated on medial optical sections using Leica LCS software. Briefly, the two-dimensional scatter plot diagram of each image was analyzed to evaluate the spatial co-localization of the fluorochromes. For each scatter plot diagram, areas with the strongest co-localizing green and red fluorescence, i.e. pixels with intensity values greater than 150 gray levels (on a scale from 0 to 255) for both detectors, were selected to calculate the co-localization maps and create a binary image (Riccio *et al.*, 2004).

Results

PKCε interacts with MATER in MATER$^+$-HEK293T

Scansite is a web-accessible programme that predicts interactions between proteins using experimental binding data from peptide library and phage display experiments (Obenauer and Yaffe, 2004). Scansite predicts PKCε as a binding partner of human MATER protein. This prompted us to investigate whether MATER and PKCε interact *in vitro* and *in vivo*.

In order to obtain a large expression of MATER for coimmunoprecipitation assay, MATER was transiently expressed in HEK293T cells, as reported in Materials and Methods section. Figure 1A shows that the exogenous MATER was localized by anti-NALP5 antibody at the same molecular weight of MATER expressed in oocytes and cumulus cells. In fact the MATER expression vector encodes human MATER residue 25–1199, therefore the almost full length protein.

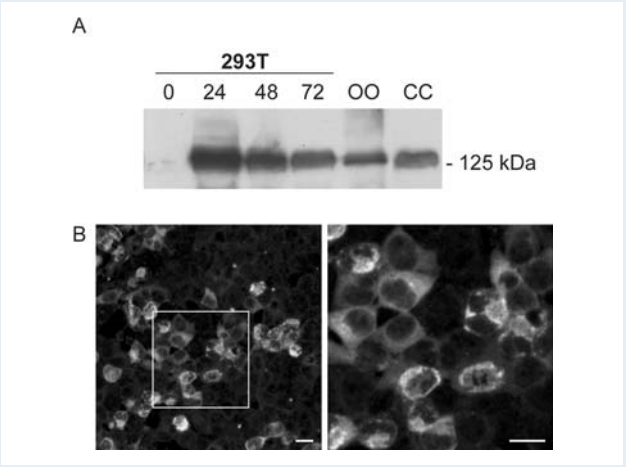

Figure 1 Expression of MATER in transfected HEK293T. (**A**) Expression vector containing full-length MATER was transfected into 293T cells. Immunoblot of *MATER*$^+$HEK293T lysates after transfection different times (0–24–48–72 h), oocytes (OO) or cumulus cells (CC). Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with antibody to MATER and visualized by chemiluminescence. Molecular masses (kDa) are indicated at the right. (**B**) Transfection efficiency test by immunofluorescence analysis of *MATER*$^+$HEK293T. Cells were fixed, permeabilized and stained with anti-MATER. Scale bar: 10 μm.

The optimal transfection time is 24 h, therefore all the experiments below will be carried out in this condition.

Immunofluorescence analysis shows the high efficiency of MATER transfection that localizes mostly in the cytoplasm of 293T cells (Fig. 1B).

To determine if the two proteins physically interact, lysates from 293T cells expressing MATER were immunoprecipitated with anti-NALP5. The immunoprecipitated proteins were separated by SDS-PAGE and analyzed by immunoblot using antibodies specific to PKCε (Fig. 2A). PKCε was detected as a 90 kDa protein in whole lysate of 293T expressing MATER or MATER/PKCε (Fig. 2A, lines 5–6) and was immunoprecipitated by anti-MATER antibody from 293T expressing MATER or MATER/PKCε (Fig. 2A, lines 2–3). The overexpression of PKCε in *MATER*$^+$HEK-293T cells improves the amount of MATER–PKCε complex (Fig. 2A, line 3). PKCε was not immunoprecipitated from 293T wild type (Fig. 2A, line 1). Moreover, reciprocal experiments using monospecific antibody to PKCε to immunoprecipitate MATER in whole cell lysates from 293T expressing MATER or MATER/PKCε were successful (Fig. 2B). MATER is clearly detectable in anti-PKCε immunoprecipitates (Fig. 2B, lines 2–3). The presence of endogenous PKCε in the IP with anti-MATER and *vice versa* suggested that the two proteins interact *in vivo*.

For confocal analysis 293T transiently expressing MATER were permeabilized and stained over-night with specific antibody to PKCε and to MATER. Each antibody was raised in a different species and did not cross-react with the other target protein. Merging of FITC and Cy3 signals from *MATER*$^+$HEK-293T cells double labelled with anti-MATER and anti-PKCε Abs, demonstrated the localization of both proteins in the cytoplasm of 293T cells. Colocalization binary map shows the pixels of the image in which both green and red signals are present,

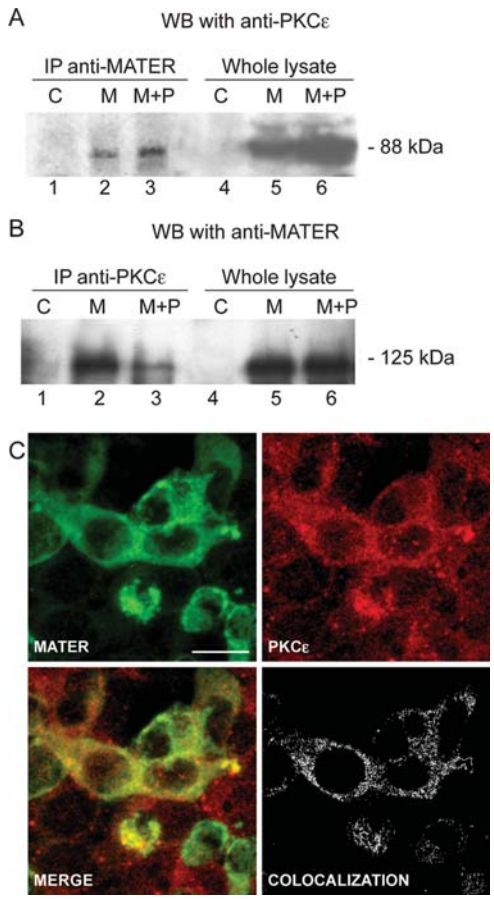

Figure 2 PKCε interacts with MATER in transfected HEK293T. (**A**) Immunoprecipitation of PKCε with antibodies to MATER. 293T wild type, *MATER*$^{+}$ and *MATER*$^{+}$/PKCε^{+} were lysed (0.5% Triton X100 in PBS) and either analysed directly (lines 4,5,6) or immunoprecipitated with anti-MATER (lines 1,2,3). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, incubated with anti-PKCε and visualized with chemiluminescence. WB = western blot. (**B**) Immunoprecipitation of MATER with antibodies to PKCε. The samples of Fig.2A were lysed (0.5% Triton X100 in PBS) and either analysed directly (lines 4,5,6) or immunoprecipitated with anti-PKCε (lines 1,2,3). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, incubated with anti-MATER and visualized with chemiluminescence. Molecular masses (kDa) are indicated at the right of both A and B. The data shown are representative of three independent experiments. (**C**) Co-localization of MATER and PKCε by confocal microscopy. *MATER*$^{+}$HEK293T cells were stained with anti-MATER (green) and anti-PKCε (red). Co-localization binary map (black & white) of PKCε and MATER signals was determined. Scale bar: 10 μm.

thus indicating around the 40% of the signal from MATER colocalize with the signal from endogenous PKCε (Fig. 2C).

MATER is phosphorylated by PKCε *in vitro*

Scansite programme indicates the Serine 331 as MATER's site of interaction (phosphorylation site) with this basophilic serine/threonine kinase, PKCε. Furthermore, immunoblot experiments by Ab directed

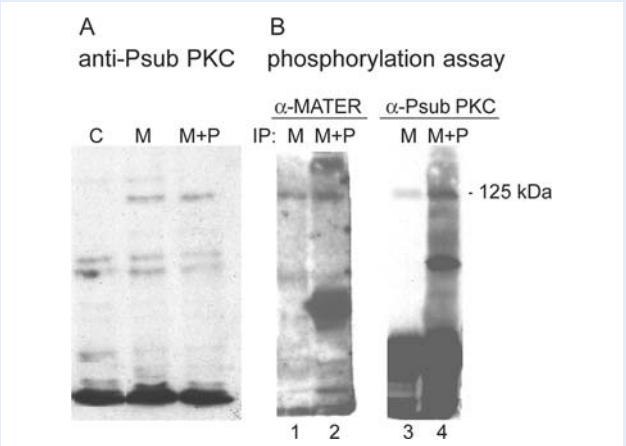

Figure 3 PKCε phosphorylates MATER in transfected HEK293T. (**A**) 293T cells wild type (C) or expressing MATER (M) or MATER/PKCε (M+P) were lysed, separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-PKC phosphosubstrate. The result is representative of three independent experiments. (**B**) MATER expressed in 293T cells was immunoprecipitated with an anti-MATER antibody, then phosphorylated *in vitro* in the presence (M+P) or not (M) of PKCε-IP and ATP. Pellets were resolved on SDS-polyacrylamide gel and stained with anti-MATER (α-MATER lines 1,2) and anti-PKC phosphosubstrate (α-PsubPKC lines 3,4). The data shown are representative of three independent experiments.

against the substrates phosphorylated by PKC (Phospho-(Ser) PKC substrate antibody) show a 125 kDa band corresponding to MATER protein (Fig. 3A). This data suggests that MATER could be a phosphosubstrate of PKCε, as was then confirmed by *in vitro* phosphorylation experiments below.

In vitro phosphorylation assay was performed as reported in Materials and Methods section. In particular, MATER-IP, obtained from 293T cells transiently transfected only with MATER, was treated for 15 min with the phosphorylation buffer in presence (M+P) or not (M) of PKCε-IP. Samples were run on SDS-polyacrylamide gel and stained with anti-MATER and anti-Phospho-(Ser) PKC substrate antibody.

Figure 3B shows that MATER is clearly detectable in both the IP samples and that, after anti-Phospho-(Ser) PKC Substrate incubation, a weak phosphorylation of MATER is detected in the M sample, most probably due to endogenous PKCε which, as shown above, co-immunoprecipitates with MATER. In addition, the band corresponding to 125 kDa becomes more intense in the sample (M+P) in which MATER IP have been exposed to phosphorylation buffer with PKCε IP (Fig. 3B line 4).

Effect of MATER expression on phosphorylation pathways in HEK293T cells

Further experiments on HEK-293T cells expressing MATER show that the presence of MATER protein induces an increase of PKCε amount, confirming the cross-talk between the expression of these two proteins (Fig. 4A left panel).

PKC has served as the archetypal lipid-regulated kinase, providing a prototype for lipid-controlled kinase activation that is followed by

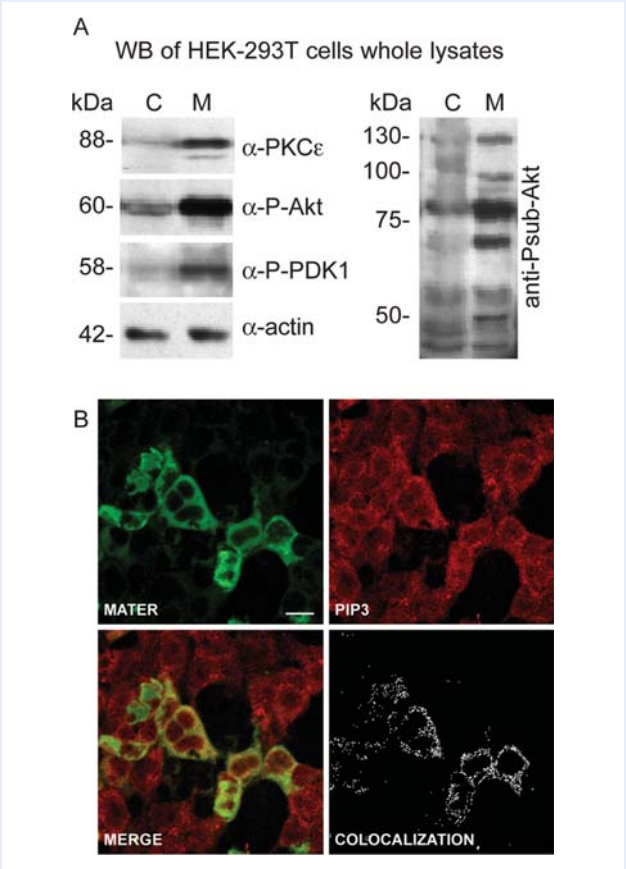

Figure 4 Effect of MATER expression on phosphorylation pathways in transfected HEK293T cells.
(**A**) Western blot analysis of whole lysates of 293T wild type or expressing MATER. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, incubated with the indicated antibodies and visualized with chemiluminescence. The data shown are representative of at least three independent experiments. (**B**) Co-localization of MATER and PIP_3 by confocal microscopy. *MATER*$^+$ HEK293T cells were stained with anti-MATER (green) and anti-PIP_3 (red). Binary map (black & white) of co-localized PIP_3 and MATER signals was determined. Scale bar: 10 μm.

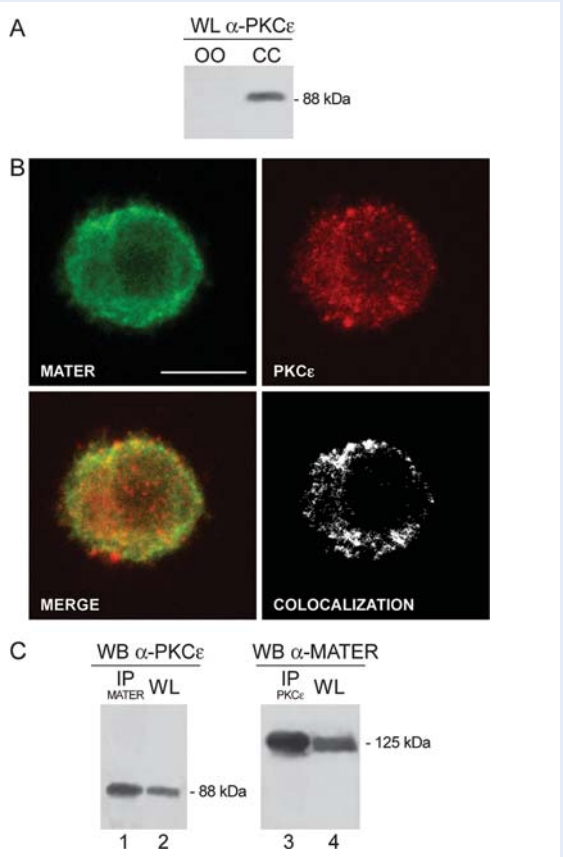

Figure 5 PKCε interacts with MATER in cumulus cells.
(**A**) PKCε expression in oocytes and cumulus cells. Western blot analysis of whole lysates of human oocytes and cumulus cells were resolved by SDS-PAGE, transferred to nitrocellulose, incubated with anti-PKCε and visualized with chemiluminescence. The data shown are representative of three independent experiments. (**B**) Double labelling of human cumulus cells with anti-MATER (green) and anti-PKCε (red). Co-localization binary map (black & white) of MATER and PKCε signals was determined. Scale bar: 10 μm. (**C**) Cumulus cells were lysed (0.5% Triton X100 in PBS) and either analyzed directly (lines 2,4 WL) or immunoprecipitated with anti-MATER (line 1 IP_{MATER}) or anti-PKCε (line 3 $IP_{PKCε}$). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, incubated with anti-PKCε (lines 1,2) anti-MATER (lines 3,4) and visualized with chemiluminescence. Molecular masses (kDa) are indicated at the right of both A and C. The data shown are representative of three independent experiments.

kinases throughout the kinome, including its close cousin, Akt (protein kinase B). PKC and Akt transduce signals propagated by the two major lipid second messenger pathways in cells, those of diacylglycerol signalling and phosphatidylinositol-3,4,5-trisphosphate (PIP_3) signalling, respectively.

In fact, PIP_3 is another interesting lipid signalling messenger suggested to interact with MATER with high stringency by Scansite programme. PIP_3-binding Pleckstrin homology (PH) domain could associate with MATER. Colocalization immunofluorescence experiments shown in Fig. 4B suggest that association indeed occurs.

MATER expression could also modulate Akt pathways. Notably, members of the Akt family of kinases contain a PH domain that selectively recognizes PIP_3, thus serving as one of the major mechanisms to transduce signals that activate PI3 kinase. In fact, a phosphorylation increase of Akt and PDK1, the upstream kinase for Akt containing a PH domain, occurs in *MATER*$^+$HEK-293T cells (Fig. 4A left panel). Indeed, whole lysates of cells expressing MATER present higher intensity level of Akt phospho-substrates than control cells (Fig. 4A right panel).

PKCε–MATER interaction in human cumulus cells

To determine if the two proteins can interact in cells physiologically expressing MATER, at first we performed immunoblot analysis of lysates obtained from human oocytes and cumulus cells, in order to check the expression of PKC isoforms. As reported in the literature, some of the conventional PKC isoforms are present in both the

cells types (data not shown), but the band corresponding to PKCε, member of the novel PKC family, is clearly visible only in the cumulus sample, although in human oocyte line is not detectable (Fig. 5A).

On the basis of this result and of data recently published by our group demonstrating the MATER is also expressed in cumulus cells (Sena *et al*., 2009), we investigated the possible interaction between MATER and PKCε only in human isolated cumulus cells.

Cumulus cells collected by pick-up technique during clinical *in vitro* fertilization procedures, were isolated, permeabilized and stained with specific antibodies to PKCε and to MATER. Confocal analysis demonstrated the co-localization of the two proteins in the cytoplasm of this follicular cell population (Fig. 5B).

To determine if the two proteins physically interact, immunoprecipitation assay was performed as described above. The specific antibodies to MATER and PKCε were used to immunoprecipitate whole cell lysates from cumulus cells. The immunoprecipitated material was separated by SDS-PAGE and analysed by immunoblot (Fig. 5C). A band recognized by PKCε antibody is visible in cumulus cells lysate and in anti-MATER IP (Fig. 5C, lines 1–2). MATER was detected as a 125 kDa protein in cumulus lysate (Fig. 5C, line 4) and was immunoprecipitated by anti-PKCε antibody (Fig. 5C, line 3).

All the data together confirm the suggestion that the interaction, observed in HEK293T cells expressing MATER, occurs similarly in cumulus cells under physiological conditions.

Discussion

Mammalian oocytes mature in a stepwise manner through ovarian folliculogenesis, ultimately reaching competence to complete oocyte maturation at the final stage of Graafian follicle development. The fully-grown oocyte is tightly surrounded by compact layers of specialized granulosa cells (cumulus cells) to form a cumulus–oocyte complex (COC). Cellular events that immediately occur in COCs in the ovulatory phase are strictly regulated by pituitary hormones, steroids, growth factors and so on (Kimura *et al*., 2007). Knowledge of the efficient mechanisms and the downstream cascades of the key molecules controlling follicular maturation may gradually lead to improvement of the present oocyte/embryo culture systems and gamete biotechnology.

There is now increasingly ample molecular evidence that maternal effect genes are crucial in preimplantation (Christians *et al*., 2000; Gurtu *et al*., 2002; Wu *et al*., 2003; Payer *et al*., 2003; Burns *et al*., 2003; Bortvin *et al*., 2004; Ma *et al*., 2006; Bultman *et al*., 2006; Nakamura *et al*., 2007) and post-implantation (Bourc'his *et al*., 2001; Howell *et al*., 2001; Leader *et al*., 2002; Ye *et al*., 2005) development.

The human *MATER* gene has been well characterized and the protein is clearly detectable in oocytes (Tong *et al*., 2002) and in cumulus cells (Sena *et al*., 2009) but the physiological role during human folliculogenesis has yet to be characterized. The 1111 amino acid cytoplasmic protein contains a NACHT (NTPase) domain (Koonin and Aravind, 2000) and two repeat motifs near its termini. A five tandem hydrophilic repeat (18 amino acids) at the amino terminus of MATER has homology with dentin matrix protein 1 (George *et al*., 1993), and a 14 tandem LRR (28–29 amino acids) near the carboxyl terminus (Tong *et al*., 2000b) is a motif implicated in protein–protein interactions (Kobe and Kajava, 2001).

Therefore, we set out to investigate the role of MATER protein in human follicle. The localization and the interaction of MATER was analysed by indirect immunofluorescence, immunoblot and coimmunoprecipitation experiments in both *MATER*$^{+}$HEK293T and isolated oocytes and cumulus cells after ovarian stimulation for *in vitro* fertilization.

Here we show that *in vitro* MATER expression leads to phosphorylation pathways activation. In particular, we observed that MATER interacts with phosphatidylinositol-3,4,5-trisphosphate (PIP_3) and Akt signalling is stimulated in *MATER*$^{+}$HEK293T.

Protein kinases transduce signals emanating from the plasma membrane, thus lipid second-messenger regulated kinases contain one or more membrane-targeting modules whose membrane engagement results in protein kinase activation. As reported in review by Newton, notably members of the Akt family of kinases contain a PH domain that selectively recognizes PIP_3, thus serving as one of the major mechanisms to transduce signals that activate PI3 kinase. PDK1, the upstream kinase for most of the AGC kinases (protein kinase A/protein kinase G/PKC) also has a PH domain; although it binds phosphoinositides with high affinity, this kinase is constitutively active in cells, with substrate conformation controlling down-stream signalling (Newton, 2009). Figure 5 shows that when MATER is expressed PIP_3 co-localizes with MATER and an increase in PDK1 and Akt phosphorylation occurs, suggesting that MATER, that is expressed specifically in oocytes and cumulus cells (Sena *et al*., 2009), could be involved in coordinating PI3-K/Akt pathway activation.

These effects can be explained by recent studies implying that the PI3-K/Akt pathway and the mitogen activated protein kinase (MAPK) pathway contribute to the progress of follicle stimulating hormone (FSH)-induced meiosis in mice (Kimura *et al*., 2007). Thus, high activity of the PI3-kinase/Akt pathway in cumulus cells plays an important role in FSH regulation of cell function. It is estimated that PI3-kinase in cumulus cells is required for both the suppression of spontaneous meiotic resumption and the induction of gonadotrophin-stimulated meiotic resumption (Shimada *et al*., 2003).

Furthermore, here we have demonstrated for the first time that MATER can be also a phosphorylation substrate of the isoform ε of PKC, indicating that MATER can play an active role in signal transduction from the plasma membrane to the cytoplasm. This interaction occurs both in *MATER*$^{+}$HEK293T cells and in cumulus cells. In contrast PKCε is not expressed in human oocytes.

PKC, a family of serine/threonine kinases, plays an important role in the growth factor signal transduction pathway. PKCε, a member of the novel PKC group, is believed to function as an antiapoptotic protein. PKCε collaborates with other signalling pathways, such as Ras/Raf/ERK and Akt, to regulate cell survival and cell death. Conventional and novel PKC isozymes, lipid second messenger-sensing Ser/Thr protein kinases, contain diacylglycerol-binding C1 domains and transduce signals that trigger diacylglycerol production. Moreover, the PH domain of Akt binds agonist-produced PIP_3, an event that exposes the activation loop for phosphorylation by PDK1, the same upstream kinase that modifies PKC; this phosphorylation is rapidly followed by phosphorylation of the hydrophobic motif. Once fully-phosphorylated, Akt is locked in an active conformation and diffuses throughout the cell phosphorylating down-stream substrates (see review by Newton, 2009). There is considerable evidence for PKCε-mediated increase

in Akt phosphorylation and activity (Basu and Sivaprasad, 2007). Increased Akt activity may be required to mediate the antiapoptotic effects of PKCε. Interestingly, signalling via both proteins was required for efficient MAPK activation, suggesting that the PKCε–Akt complex can cross-talk with a third pathway to mediate its antiapoptotic effects. PKCε may also enhance Akt activity indirectly (Newton, 2009). The indirect regulation of Akt by PKCε can involve integrins or secretion of growth factors that can elicit signalling through the pro-survival PI3-K pathway (Basu and Sivaprasad, 2007). Notably, the rate of proliferation of cumulus cells has been suggested to correlate with implantation potential in human assisted reproduction (Gregory, 1998). Therefore a proper MATER expression during follicular maturation could be crucial for pro-survival signal transduction, such as PI3-K/Akt/PKCε pathways.

Acknowledgements

The authors thanks to Dr L.M. Nelson and Dr Z.B. Tong for the gift of the human MATER expression vector.

Funding

This work was supported by grants from Fondazione Cassa di Risparmio di Modena, Fondazione Manodori and Regione Emilia Romagna, programma di ricerca Università. For this study was utilized the confocal microscope Leica TCS SP2 of the C.I.G.S. (Centro Interdipartimentale Grandi Strumenti) of University of Modena and Reggio Emilia, financed from Fondazione Cassa di Risparmio di Modena.

References

Basu A, Sivaprasad U. Protein kinase Cepsilon makes the life and death decision. *Cell Signal* 2007;**19**:1633–1642.

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.

Bourc'his D, Xu GL, Lin CS, Bollman B, Bestor TH. Dnmt3L and the establishment of maternal genomic imprints. *Science* 2001; **294**:2536–2539.

Bultman SJ, Gebuhr TC, Pan H, Svoboda P, Schultz RM, Magnuson T. Maternal BRG1 regulates zygotic genome activation in the mouse. *Genes Dev* 2006;**20**:1744–1754.

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.

Cecconi S, Ciccarelli C, Barberi M, Scaldaferri L, Canipari R. Granulosa cell-oocyte interaction. *Obstet Gynecol* 2004;**115S**:S19–S22.

Cenni V, Sirri A, Riccio M, Lattanzi G, Santi S, De Pol A, Maraldi NM, Marmiroli S. Targeting of the Akt/PKB kinase to the actin skeleton. *Cell Mol Life Sci* 2003;**60**:2710–2720.

Christians E, Davis AA, Thomas SD, Benjamin IJ. Maternal effect of hsf1 on reproductive success. *Nature* 2000;**407**:693–694.

Damiano JS, Oliveira V, Welsh K, Reed JC. Heterotypic interactions among NACHT domains: implications for regulation of innate immune responses. *Biochem J* 2004;**381**:213–219.

Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. *Reproduction* 2001;**122**:829–838.

Eppig JJ, Chesnel F, Hirao Y, O'Brien MJ, Pendola FL, Watanabe S, Wigglesworth K. Oocyte control of granulosa cell development: how and why. *Hum Reprod* 1997;**12**:127–132.

Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW *et al.* Mutations in an oocyte-derived growth factor gene (BMP 15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nat Genet* 2000;**25**:279–283.

George A, Sabsay B, Simonian PA, Veis A. Characterization of a novel dentin matrix acidic phosphoprotein. Implications for induction of biomineralization. *J Biol Chem* 1993;**268**:12624–12630.

Gilchrist RB, Ritter LJ, Armstrong DT. Oocyte-somatic cell interactions during follicle development in mammals. *Anim Reprod Sci* 2004;**82–83**:431–446.

Gregory L. Ovarian markers of implantation potential in assisted reproduction. *Hum Reprod* 1998;**4**:117–132.

Gumucio D, Diaz A, Schaner P, Richards N, Babcock C, Schaller M, Cesena T. Fire and ICE: the role of pyrin domain-containing proteins in inflammation and apoptosis. *Clin Exp Rheumatol* 2002;**20**:45–53.

Gurtu VE, Verma S, Grossmann AH, Liskay RM, Skarnes WC, Baker SM. Maternal effect for DNA mismatch repair in the mouse. *Genetics* 2002; **160**:271–277.

Harton JA, Linhoff MW, Zhang J, Ting JP. Cutting edge: CATERPILLER: a large family of mammalian genes containing CARD, pyrin, nucleotide-binding, and leucine-rich repeat domains. *J Immunol* 2002; **169**:4088–4093.

Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nat Genet* 2001;**29**:301–305.

Howell CY, Bestor TH, Ding F, Latham KE, Mertineit C, Trasler JM, Chaillet JR. Genomic imprinting disrupted by a maternal effect mutation in the Dnmt1 gene. *Cell* 2001;**104**:9–838.

Hutt KJ, Albertini DF. An oocentric view of folliculogenesis and embryogenesis. *Reprod BioMed Online* 2007;**14**:758–764.

Kajava AV. Structural diversity of leucine-rich repeat proteins. *J Mol Biol* 1998;**277**:519–527.

Kimura N, Hoshino Y, Totsukawa K, Sato E. Cellular and molecular events during oocyte maturation in mammals: molecules of cumulus–oocyte complex matrix and signalling pathways regulating meiotic progression. *Soc Reprod Fertil Suppl* 2007;**63**:327–342.

Kobe B, Kajava AV. The leucine-rich repeat as a protein recognition motif. *Curr Opin Struct Biol* 2001;**11**:725–732.

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.

Laitinen M, Vuojolainen K, Jaatinen R, Ketola I, Aaltonen J, Lehtonen E, Heikinheimo M, Ritvos O. A novel growth differentiation factor-9 (GDF-9) related factor is co-expressed with GDF-9 in mouse oocytes during folliculogenesis. *Mech Dev* 1998;**78**:135–140.

Latham KE. Mechanism and control of embryonic genome activation in mammalian embryos. *Int Rev Cytol* 1999;**193**:71–124.

Leader B, Lim H, Carabatsos MJ, Harrington A, Ecsedy J, Pellman D, Maas R, Leder R. Formin-2, polyploidy, hypofertility and positioning of the meiotic spindle in mouse oocytes. *Nat Cell Biol* 2002;**4**: 921–928.

Lee FS, Vallee BL. Structure and action of mammalian ribonuclease (angiogenin) inhibitor. *Prog Nucleic Acid Res Mol Biol* 2006;**81**:1–49.

Li L, Baibakov B, Dean J. A subcortical maternal complex essential for preimplantation mouse embryogenesis. *Dev Cell* 2008;**15**:416–425.

Ma J, Zeng F, Schultz RM, Tseng H. Basonuclin: a novel mammalian maternal-effect gene. *Development* 2006;**133**:2053–2062.

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–R120.

Nakamura T, Arai Y, Umehara H, Masuhara M, Kimura T, Taniguchi H, Sekimoto T, Ikawa M, Yoneda Y, Okabe M *et al.* PGC7/Stella protects against DNA demethylation in early embryogenesis. *Nat Cell Biol* 2007;**9**:64–71.

Newton AC. Lipid activation of protein kinases. *J Lipid Res* 2009; **50**:S266–S271.

Obenauer JC, Yaffe MB. Computational prediction of protein–protein interactions. *Methods Mol Biol* 2004;**261**:445–468.

Pawlowski K, Pio F, Chu ZL, Reed JC, Godzik A. PAAD-A new protein domain, associated with apoptosis, cancer and autoimmune diseases. *Trends Biochem Sci* 2001;**26**:85–87.

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.

Riccio M, Dembic M, Cinti C, Santi S. Multifluorescence labelling and colocalization analyses. In: Giordano A, Romano G (eds). *Methods in Molecular Biology: Cell Cycle Control and Dysregulation Protocols*, **Vol. 285**, NY: Humana Press Inc, 2004, 171–177.

Sena P, Riccio M, Marzona L, Nicoli A, Marsella T, Marmiroli S, Bertacchini J, Fano RA, La Sala GB, De Pol A. Human MATER localization in specific cell domains of oocytes and follicular cells. *Reprod Biomed Online* 2009;**18**:226–234.

Shimada M, Ito J, Yamashita Y, Okazaki T, Isobe N. Phosphatidylinositol 3-kinase in cumulus cells is responsible for both suppression of spontaneous maturation and induction of gonadotropin-stimulated maturation of porcine oocytes. *J Endocrinol* 2003;**179**:25–34.

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* 2000a;**26**: 267–268.

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* 2000b;**11**:281–287.

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.

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.

Tschopp J, Martinon F, Burns K. NALPs: a novel protein family involved in inflammation. *Nat Rev Mol Cell Biol* 2003;**4**:95–104.

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.

Ye X, Hama K, Contos JJ, Anliker B, Inoue A, Skinner MK, Suzuki H, Amano T, Kennedy G, Arai H *et al.* LPA3-mediated lysophosphatidic acid signalling in embryo implantation and spacing. *Nature* 2005; **435**:104–108.

Submitted on May 5, 2009; resubmitted on June 16, 2009; accepted on June 16, 2009