Molecular Human Reproduction, Vol.21, No.4 pp. 320–329, 2015
Advanced Access publication on December 26, 2014 doi:10.1093/molehr/gau116

molecular human reproduction

ORIGINAL RESEARCH

Identification of a human subcortical maternal complex

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Submitted on September 30, 2014; resubmitted on December 2, 2014; accepted on December 19, 2014

ABSTRACT: Maternal effect genes play essential roles in early embryonic development. However, the mechanisms by which maternal effect genes regulate mammalian early embryonic development remain largely unknown. Recently, we identified a subcortical maternal complex (SCMC) that is composed of at least four proteins encoded by *Mater*, *Floped*, *Tle6* and *Filia* and is critical for mouse preimplantation development. The present study demonstrates that human SCMC homologous genes (*NLRP5*, *OOEP*, *TLE6* and *KHDC3L*) are specifically expressed in the oocytes of human fetal ovaries. The proteins of this complex co-localize in the subcortex of human oocytes and early embryos. Furthermore, the SCMC proteins physically interact with each other when they are co-expressed in cell lines. These results indicate that human NLRP5, OOEP, TLE6 and KHDC3L function as a complex in the oocytes and early embryos of *Homo sapiens*. Considering the important roles of the SCMC in mouse early embryogenesis, the characterization of the human SCMC will provide a basis for investigating human early embryonic development and will have clinical implications in human female infertility or recurrent spontaneous abortion.

Key words: development / maternal factor genes / SCMC / *NLRP5* / female infertility

Introduction

During mammalian follicular development, along with a concomitant ~300-fold increase in volume, oocytes accumulate a substantial amount of maternal RNAs and proteins (Schultz *et al.*, 1979; Sternlicht and Schultz, 1981; Wassarman, 1994; Liu *et al.*, 2006). Because transcriptional activity is quiescent before zygotic genome activation, early embryonic development relies almost entirely on the maternally stored macromolecules. Maternal effect genes were described in *Drosophila* as early as 1980 (Nusslein-Volhard *et al.*, 1980) and have been extensively studied in other animal models (Morisato and Anderson, 1995; Bowerman, 1998; Dosch *et al.*, 2004; Wagner *et al.*, 2004; Heasman, 2006). However, in mammals, maternal effect genes were not identified until 2000 (Christians *et al.*, 2000; Tong *et al.*, 2000). Although mouse genetics is a powerful tool for studying the maternal effect genes, very few maternal effect genes (<30) have been identified in mammals (Li *et al.*, 2010, 2013). In 2006, the first human maternal effect gene was reported through screening for the mutation responsible for familial biparental hydatidiform mole (FBHM), an extremely rare human maternal recessive disease (Murdoch *et al.*, 2006). Currently, only two maternal effect genes (*NLRP7* and *KHDC3L*) have been characterized in *Homo sapiens*. *NLRP7* and *KHDC3L* have been shown to be associated with FBHM (Parry *et al.*, 2011), but the molecular mechanisms underlying this human disorder remain largely unclear (Nguyen and Slim, 2014).

Recently, we identified a subcortical maternal complex (SCMC) in mouse oocytes and early embryos, and it is composed of at least four proteins: MATER (Maternal Antigen That Embryos Require), Filia (designated as a daughter of MATER, mother in Latin), FLOPED (Factor Located in Oocytes Permitting Embryonic Development) and TLE6 (Transducin-Like Enhancer of Split 6). *Mater* (official name *Nlrp5*) encodes a protein with 1163 amino acids, including LRR and PYD domains, and was originally identified as an antigen-associated gene in a mouse model of autoimmune oophoritis. This protein was later serendipitously revealed as one of the first maternal effect genes in mammals (Tong and Nelson, 1999; Tong *et al.*, 2000). *Floped* (official name *Ooep*), the founding member of the SCMC, was identified in a screen for oocyte-specific proteins and encodes a 164-amino acid protein that includes an atypical KH domain (Herr *et al.*, 2008; Li *et al.*, 2008). *Filia* (official name *Khdc3*, encoding a protein with 346 amino acids), along with *Khdc1*, *Dppa5*, and *Floped*, has been classified into a new superfamily of oocyte/embryo-expressed genes that are characterized by a FILIA-N-Like domain (Pierre *et al.*, 2007; Wang *et al.*, 2012). *Tle6* (encoding 581 amino acids), a member of the Groucho/TLE transcriptional co-repressor gene family, has been identified as *Grg6*, one of the targets of E2A-HLF (hepatic leukemia factor) (Dang *et al.*, 2001).

The genes of SCMC components are transcribed and accumulate during oogenesis, but they play only minor roles before early embryonic development. The disruption of single copies of the genes encoding *Mater*, *Floped* or *Tle6* does not affect oogenesis, ovulation or fertilization, but it produces female infertility with the absence of the SCMC and arrested embryonic development at the cleavage stage (Tong *et al.*, 2000; Li *et al.*, 2008; Tashiro *et al.*, 2010; Yu *et al.*, 2014). *Filia*-null female mice have the normal SCMC and exhibit a subtle phenotype, with impaired fertility resulting from chromosome instability and the high incidence of aneuploidy in Filia-null embryos (Zheng and Dean, 2009). The various phenotypes of these mouse lines are consistent with the dependence of SCMC on FLOPED, MATER and TLE6, but not Filia, suggesting that these maternal effect genes are tightly regulated and function as an entire protein complex (Li *et al.*, 2008; Zheng and Dean, 2009; Yu *et al.*, 2014). Most recently, we have demonstrated that FLOPED, MATER and TLE6 play an important role as a complex and control spindle position to ensure symmetrical cell division by regulating the dynamics of F-actin in the mouse zygote (Yu *et al.*, 2014).

All SCMC proteins have homologues in mammals, including human. Compared with mouse SCMC proteins, the human homologues (*NLRP5*, *OOEP*, *KHDC3L* and *TLE6*) share 46% identity for MATER, 39% identity for FLOPED, 41% identity for FILIA and 44% identity for TLE6 (Li *et al.*, 2008). Human *NLRP5* has been cloned and characterized based on similarities in the oocyte-specific expression pattern and sequences, suggesting that this gene may have analogous functions in human and mouse (Tong *et al.*, 2000, 2002). NLRP5 was also identified as a potential autoantigen involved in hypoparathyroidism in patients with autoimmune polyglandular syndrome type I (Alimohammadi *et al.*, 2008). *KHDC3L* (also known as *c6orf221* and *ECAT1*) was identified as the second maternal effect gene involved in FBHM, and the expression of *KHDC3L* mRNA was detected in human oocytes (Parry *et al.*, 2011). Although it is well established that MATER, FLOPED, FILIA and TLE6 are mostly oocyte- and early embryo-specific genes and function as components of the SCMC in mouse early embryogenesis (Li *et al.*, 2008; Ohsugi *et al.*, 2008; Zheng and Dean, 2009), much less is known regarding these genes and the SCMC complex in other species.

In the current study, we investigated the gene expression profiles of *OOEP*, *TLE6*, *NLRP5* and *KHDC3L* in human fetal tissues and documented their protein localization in human oocytes and embryos. Furthermore, we studied the interactions between the human SCMC proteins by expressing these genes in cell lines. Our results indicate that human OOEP, TLE6, NLRP5 and KHDC3L form the SCMC and may play important roles during early embryonic development in *Homo sapiens*.

Materials and Methods

Collection of human tissues and embryos

The methods applied in this study complied with the Helsinki Declaration. The use of human tissues and embryos for this study was approved by the Ethics Committee of Peking University Third Hospital (2011SZ013). Tissues of fetuses at gestational ages 19–22 weeks used in this study were obtained after legal voluntary termination of pregnancy for maternal physical disorders (severe pre-eclampsia with hypertension and proteinuria) according to the Chinese legislation that allows termination of pregnancy for medical reasons. The oocytes were from IVF processes during which the oocytes were considered as the failure of fully maturation after hormonal stimulation and could not used for clinical treatment. The human embryos used for this study were clinically determined as showing abnormal development and therefore could not be used for IVF processes. Before we procured the human tissues, oocytes or embryos, informed consent for permission for scientific use of these samples were obtained from the donors, in accordance with the guidelines outlined by the Committee of Peking University Third Hospital. We used oocytes that did not fully mature and embryos which were polyspermy and/or arrested at diverse stages after abnormal fertilization in IVF processes for this study.

RNA isolation and real-time RT-PCR

Total RNA was extracted from the tissues of two fetuses at gestational ages 19 weeks with RNAzol®RT (Molecular Research Company). After treatment with DNase I (Promega), 500 ng RNA was used as a template in a 10-μl reverse transcription reaction (37°C for 15 min) containing 50 pmol random hexamers, 25 pmol Oligo dT primer and 5 U of Prime Script RT Enzyme Mix I (Takara). Each 10-μl reaction contained 5 μl 2× SYBR Green Mix (Takara), 0.2 μl 10 μM solution of each primer, 0.2 μl ROX Dye II, 2 μl diluted cDNA and 2.4 μl sterile distilled water. The quantitative real-time PCR was performed on an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems). Each qPCR assay was performed in triplicate. The relative expression level of the tested genes was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with Prism 5 (GraphPad) and normalized to GAPDH.

KHDC3L antibody production

Primers (Forward: 5′-GGATGGACGCTCCCAGGCGG-3′ and Reverse: 5′-CGCTCATAATCTAGTAACTGG-3′) were designed to amplify the cDNA sequence encoding the full-length of human *KHDC3L*. The cDNA was cloned into the pET30-TEV-LIC Vector (Novagen) downstream of a 6xHis tag and transformed into *Escherichia coli* BL21 (DE3 strain). The cells were grown in 1 l LB medium and induced with 0.2 mM IPTG for protein expression. After the cells were sonicated on ice and centrifuged, the supernatant was loaded onto a Ni-NTA resin column (Qiagen). The protein was purified using a HiTrap Q HP column (GE Healthcare) and then loaded onto a HiLoad 16/60 Superdex-200 size-exclusion column (GE Healthcare) for further purification. A sharp peak corresponding to the target protein was pooled and concentrated to 4 mg ml^{-1}.

Preimmune serum was collected from rabbit for use as a blank control when performing western blotting after immunization. For immunization, 1 mg of purified KHDC3L [equal to ~500 μl of the antigen-complete Freund's adjuvant (Sigma) mixture] was injected into 10 subcutaneous sites on the back of the rabbit. After two rounds of boosting with antigen-incomplete Freund's adjuvant mixture, 50 ml blood was collected from the heart of the rabbit, and the immune serum was purified with a PVDF membrane.

Immunofluorescence staining paraffin sections

Human ovaries from a fetus at gestational ages 22 weeks were fixed with 4% paraformaldehyde (PFA), paraffin-embedded and sectioned (5 μm). Prior to staining, antigen retrieval was achieved by high-temperature (30 min) using 10 μM sodium citrate buffer (pH 6.0). After blocking for 1 h at room temperature with TNK buffer [0.1 M Tris–HCl, 0.55 M NaCl, 0.1 mM KCl, 0.5% BSA and 0.1% Triton X-100 in phosphate buffer saline (PBS)] containing either 1% normal goat serum (for subsequent detection of OOEP and NLRP5) or 1% normal donkey serum (KHDC3L and TLE6), the sections were incubated with anti-OOEP (Santa Cruz Biotechnology sc-241586, 1:100), anti-NLRP5 (Santa Cruz Biotechnology sc-50630, 1:100), anti-KHDC3L (homemade, 1:500) or anti-TLE6 (Abcam ab76858, 1:400) antibodies overnight at 4°C. After washing three times with PBS, the sections were incubated for 1 h at room temperature with Donkey Anti-Goat IgG

H&L (for detection of OOEP and NLRP5, Abcam ab150129, 1:500), Goat Anti-Rabbit IgG (for KHDC3L, Jackson 111-486-003, 1:500) or Donkey Anti-Mouse IgG (for TLE6, Jackson 115-547-003, 1:500). After washing with PBS (three times), the sections were stained with Hoechst 33342 dye for 10 min and mounted. Immunofluorescence imaging was performed with a Nikon ECLIPSE Ti microscope.

Immunofluorescence staining of oocytes and early embryos

Oocytes and embryos were fixed in 4% PFA for 30 min at room temperature. After permeabilization with 0.2% Triton X-100 and blocking in 5% normal donkey serum, some were incubated overnight at 4°C with anti-OOEP antibody (Santa Cruz Biotechnology sc-241586, 1:100), anti-NLRP5 antibody (Santa Cruz Biotechnology sc-50630, 1:100), anti-KHDC3L antibody (homemade, 1:500) or anti-TLE6 antibody (Abcam ab76858, 1:500). Others (1-cell embryos) were triple-stained with anti-OOEP, TLE6 and KHDC3L antibodies or NLRP5, TLE6 and KHDC3L antibodies under the same conditions. Equivalent concentrations of goat, rabbit and mouse IgG served as negative controls. After washing, stained oocytes and embryos were incubated for 1 h at room temperature with Alexa Fluor 488 conjugated Goat Anti-rabbit antibody (for KHDC3L, Jackson 111-486-003, 1:1000), Donkey Anti-Mouse antibody (for TLE6, Jackson 115-547-003, 1:1000) or Donkey Anti-Goat antibody (for OOEP and NLRP5, Abcam ab150129, 1:1000), and for triple staining, 1-cell embryos were incubated with Alexa Fluor 488 Donkey Anti-Goat antibody (Abcam ab150129, 1:1000), 549 Donkey anti-Rabbit IgG (Jackson 711-505-152, 1:1000) and 633 Goat anti-Mouse IgG (Life Technologies A-21052, 1:1000). DNA was stained with Hoechst 33342 for 10 min at room temperature, and cells were mounted. Confocal images were observed using a Zeiss LSM 780 microscope.

Coimmunoprecipitation and western blotting

Human cDNA encoding full-length of *KHDC3L*, *OOEP* and two kinds of *TLE6* (full-length *TLE6* and truncated *TLE6*) were cloned from human fetal ovary. Full-length cDNA of *NLRP5* was cloned from the pSTEC-NLRP5 plasmid (kindly provided by Dr. Nelson). The cDNA was then subcloned into pCMV-HA and pCMV-Myc eukaryotic expression vectors. Equal amount (2.5 μg) of two components with different tags of SCMC was co-transfected into HEK293T cells using 10 μl Lipofectamine 2000 reagent (Invitrogen). At 25 h post-transfection, the cells were washed twice with Dulbecco's phosphate buffered saline. In each well of a six-well plate, the cells were lysed with 500 μl IP lysis buffer (Thermo) including protease inhibitor cocktail (Roche) on ice for 30 min. The lysate was clarified by centrifugation, and 1.8 μl HA mouse MAb (Abmart, 26D11) was added. The mixture was rotated end-over-end gently for 4 h at 4°C, and then 30 μl Protein G agarose (Santa Cruz Biotechnology) was added. After another 2 h of incubation, the product precipitated with the agarose was collected by centrifugation and washed with IP lysis buffer (four times), and then 50 μl 2× SDS loading buffer was added before the sample was heated for 5 min at 95°C and later analyzed by western blot.

Samples for immunoblots were separated by NuPAGE 4–12% SDS–PAGE and transferred to PVDF membranes (Invitrogen). The blots were blocked with Superblock Blocking Buffer (Pierce, Thermo Fisher Scientific), incubated with Myc antibody (CST 2278, 1:1500) overnight at 4°C, washed 4X with PBST and incubated for 1 h at room temperature with horseradish peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (Jackson 111-035-003, 1:10000). Immunoreactivity was detected with SuperSignal West Dura Extended Duration Substrate (Pierce 34076). Images of the blots were obtained with a Chemi Doc TMXRs$^+$ gel imaging system with Image LabTM software (Bio-Rad).

Analysis of subcellular localization of co-transfected human SCMC proteins

1×10^5 HeLa cells per well were seeded onto glass coverslips in 12-well plate. After 24 h, vectors encoding pmCherry-N1 tagged NLRP5 or KHDC3L with a nuclear localization signal (KHDC3L-pmCherry-NLS or NLRP5-pmCherry-NLS) were transfected alone (1.0 μg) or paired with vectors encoding full-length TLE6-eGFP, truncated TLE6-eGFP, KHDC3L-eGFP or NLRP5-eGFP (1.0 μg each) using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's recommendations. After 24 h, the cells were collected and fixed with 4% PFA in PBS for 20 min at room temperature. After washing with PBS, the coverslips were incubated with Hoechst 33342 for 8 min at RT. Immunofluorescence staining was visualized with a Zeiss LSM 780 microscope.

The localization of fluorescent proteins in the cells was arbitrarily classified into three categories: cytoplasm, nucleus + cytoplasm and nucleus. Cells with both red and green fluorescence were randomly selected from five 400X fields, and the subcellular localization of NLRP5 and KHDC3L was quantified as the ratio of cells with a particular NLRP5 or KHDC3L localization to the total number of cells in the field.

Statistical analysis

Quantitative analyses were based on three independent biological samples and are expressed as the mean ± SEM. Statistical analyses were conducted using two-tailed Student's *t*-test in GraphPad Prism software, with *P*-values of <0.05 considered significant.

Results

mRNA expression of OOEP, KHDC3L, TLE6 and NLRP5

Tissues of two fetuses were collected at gestational ages 19–22 weeks after legal voluntary termination of pregnancy for maternal physical disorders with informed consent from the mothers, and the expression patterns of human homologs of the SCMC were determined using real-time RT-PCR. The transcript of human *NLRP5* was strongly detected in human fetal ovary (Fig. 1A), consistent with its expression in adult ovary (Tong *et al.*, 2002). Human *KHDC3L* and *OOEP* were also detected as enriched in human ovary (Fig. 1B and C). However, human *TLE6* was highly expressed not only in ovary but also in the kidney (Supplementary data, Fig. S1A). Because human *TLE6* has another truncated form lacking an alternate in-frame segment in the 5′ coding region and using a downstream start codon (Supplementary data, Fig. S1B) (NM_001143986.1 and NM_024760.2, NCBI), we designed two pairs of primers (Supplementary data, Fig. S1B, Table S1) that specifically recognized the two variants, respectively. Full-length *TLE6* was observed to be specifically expressed in human ovary (Fig. 1D), whereas the truncated *TLE6* was restricted to kidney (Fig. 1E), suggesting that the full-length but not truncated *TLE6* functions in the ovary (TLE6 will henceforth refer to the full-length form in this study, unless otherwise stated). Thus, the transcript profiles of human *NLRP5*, *KHDC3L*, *OOEP* and *TLE6* showed an ovary-specific expression pattern in human fetal tissues.

Proteins expression of OOEP, KHDC3L, TLE6 and NLRP5

To investigate the protein expression of KHDC3L, we produced rabbit anti-KHDC3L antibody from rabbits immunized with human KHDC3L

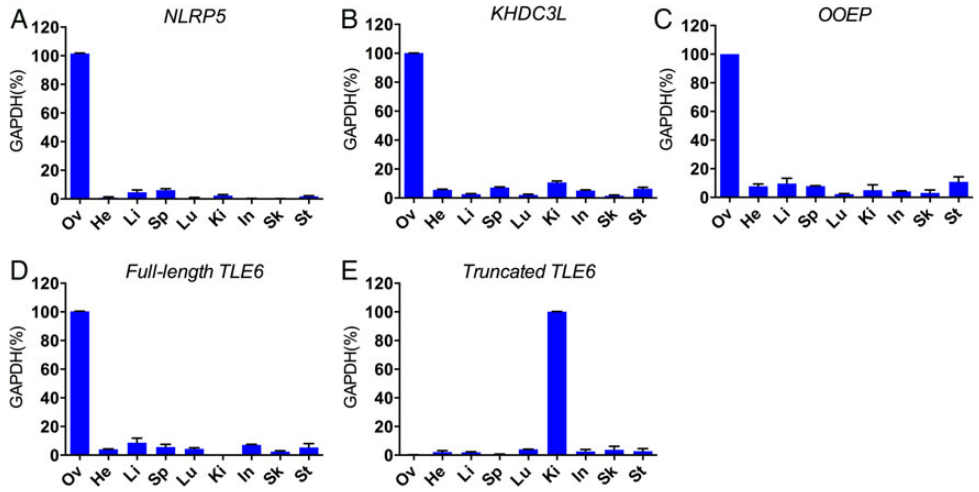


Figure 1 mRNA expression of SCMC genes in human fetal tissues. (**A**) The expression of *OOEP* in human fetal tissues. Quantitative real-time RT-PCR was performed with total RNA extracted from ovary (Ov), heart (He), liver (Li), spleen (Sp), lung (Lu), kidney (Ki), intestine (In), skin (Sk) and stomach (St) of two fetuses obtained after legal termination of pregnancy. Percentages represent relative expression levels normalized to GAPDH. (**B**) The expression of *KHDC3L* in human fetal tissues. (**C**) The expression of *NLRP5* in human fetal tissues. (**D**) The expression of full-length *TLE6* in human fetal tissues. (**E**) The expression of truncated *TLE6* in human fetal tissues detected with a specific primer localized in its exclusive N-terminus.

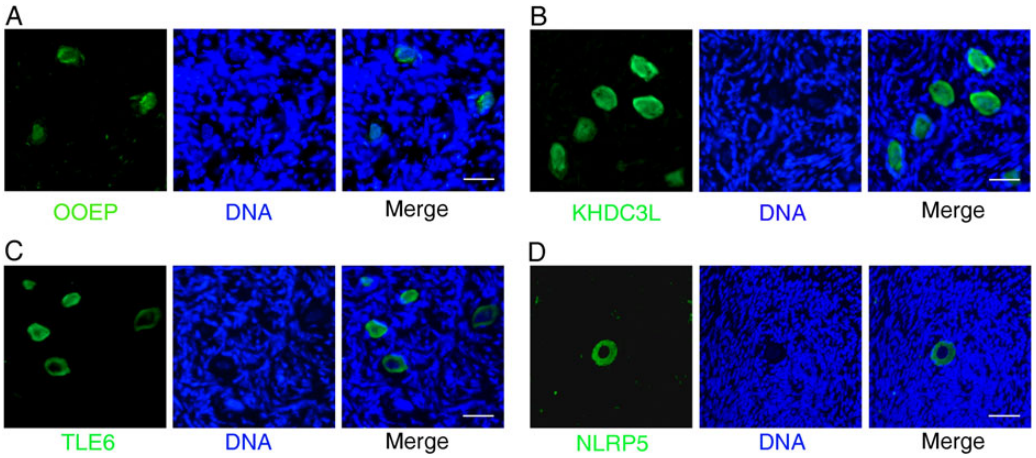


Figure 2 Protein expression of SCMC components in human fetal ovary. (**A**) Immunofluorescence of human fetal ovarian sections stained with antibody against OOEP; DNA was stained with Hoechst 33342. Scale bar, 100 μm. (**B**) Same as in (A), except with the antibody against KHDC3L. (**C**) Same as in (A), except with the antibody against TLE6. (**D**) Same as in (A), except with the antibody against NLRP5.

protein expressed in *E. coli* BL21 (Supplementary data, Fig. S2A and B). The specificity of the KHDC3L antibody was verified through western blotting and immunofluorescence staining using cells overexpressing Myc-tagged KHDC3L (Supplementary data, Fig. S2C and D). The specificity of commercial antibodies to human NLRP5 (Santa Cruz Biotechnology), OOEP (Santa Cruz Biotechnology) and TLE6 (Abcam, ab76858) was tested using methods similar to those described above (Supplementary data, Fig. S3). Because the immunogen for the anti-TLE6 antibody is recombinant human TLE6 protein (AAH20206) which included in both TLE6 and the truncated form, both TLE6 forms were recognized by this antibody when they were expressed in the cell line (Supplementary data, Fig. S3B, F and G). After verifying the specificity of these antibodies, immunofluorescence staining was used to examine the protein expression patterns of NLRP5, KHDC3L, TLE6 and OOEP in human fetal ovarian sections. The proteins of SCMC were observed to be specifically present in the oocytes and predominantly localized in the cytoplasm of human fetal ovary (Fig. 2).

Colocalization of human OOEP, KHDC3L, TLE6 and NLRP5

To localize the SCMC proteins, abnormal human oocytes and early embryos were obtained from IVF with informed consent from patients,

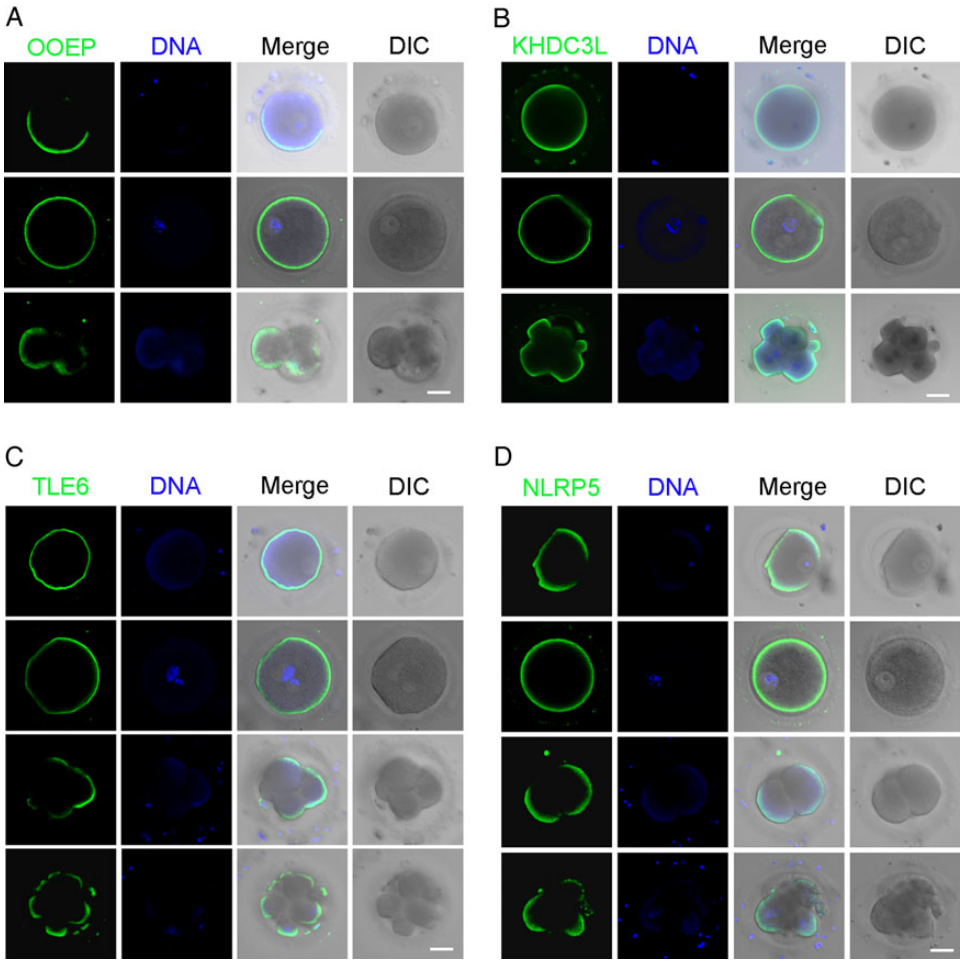


Figure 3 Localization of SCMC proteins in oocytes and early embryos. (**A**) Oocytes that failed to fertilize and human IVF embryos arrested at the 1-cell and 4-cell stages were collected, fixed and stained with anti-OOEP antibody and Hoechst, which binds to DNA. The morphology of oocytes and embryos was observed with differential interference contrast (DIC). Scale bar, 20 μm. (**B**) Same as in (A), except 1-cell and 3-cell stage embryos were stained with anti-KHDC3L. (**C**) Same as in (A), except 1-cell, 5-cell and 8-cell stage embryos were stained with anti-TLE6. (**D**) Same as in (A), except 1-cell, 2-cell and 8-cell stage embryos were stained with anti-NLRP5.

and were fixed in 4% PFA. After staining with specific antibodies, the images were obtained with confocal microscopy. NLRP5, KHDC3L, OOEP and TLE6 were observed primarily in the subcortex of oocytes and early embryos (Fig. 3). After the 2-cell stage, all of the SCMC proteins were excluded from regions of cell–cell contact in early human embryos (Fig. 3).

To further investigate whether the four proteins co-localize with each other, human zygotes were co-stained with specific antibodies to the SCMC proteins. Because the anti-OOEP and anti-NLRP5 antibodies have the same isotype (Goat), triple staining was performed with the antibodies to OOEP, KHDC3L and TLE6 or antibodies to NLRP5, KHDC3L and TLE6. OOEP, KHDC3L and TLE6 were detected to strongly co-localize in the subcortex of human zygotes (Fig. 4A). NLRP5, KHDC3L and TLE6 were also observed to be co-localized in the same region of human zygotes (Fig. 4B). Thus, we concluded that the four SCMC proteins co-localize in the subcortex of early human embryos.

Physical interaction between OOEP, TLE6, KHDC3L and NLRP5

To investigate the physical interactions of OOEP, NLRP5, TLE6 and KHDC3L, coimmunoprecipitation (Co-IP) experiments were performed by using cell lines expressing these proteins. KHDC3L, OOEP and both forms of TLE6 (the truncated form may be a negative control if it does not interact with other proteins) were Myc-tagged and co-transfected with pCMV-HA vector as the Co-IP negative control (Supplementary data, Fig. S4). Vectors for NLRP5-HA and each of the other four proteins tagged with Myc were co-transfected into HEK293T cells. The cell lysates were incubated with anti-HA to immunoprecipitate NLRP5, and potential binding partners were detected by immunoblots of the precipitate with antibody to Myc. KHDC3L, OOEP and full-length TLE6, but not truncated TLE6, were specifically precipitated by NLRP5 (Fig. 5A). Reciprocal co-transfections with NLRP5-Myc and each of the other three expression vectors with HA

tags further confirmed the interactions of OOEP-NLRP5, KHDC3L-NLRP5 and TLE6-NLRP5 (Fig. 5B). Similar experiments demonstrated the OOEP-TLE6, OOEP-KHDC3L (Fig. 5C and D), KHDC3L-OOEP and KHDC3L-TLE6 (Fig. 5E and F) interactions. All of the experiments showed the truncated TLE6 did not interact with other SCMC proteins (Fig. 5A, B and F). Thus, NLRP5, KHDC3L, OOEP and TLE6, but not truncated form, physically interact with each other when they are expressed in cell lines, and the truncated TLE6 is a good negative control for the protein interaction experiments.

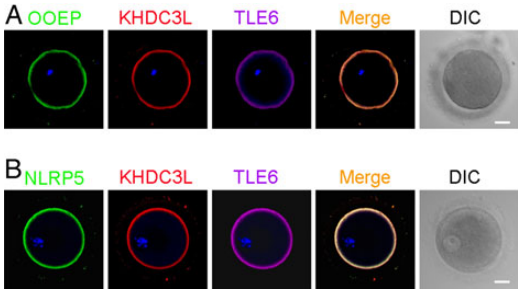


Figure 4 Co-localization of the SCMC proteins in human zygotes. (**A**) Human 1-cell stage embryos were collected, fixed and stained with antibodies against OOEP, KHDC3L and TLE6. Colocalization of OOEP, KHDC3L and TLE6 is shown in the merged image. Scale bar, 20 μm. (**B**) Same as (A), except with antibodies against NLRP5, KHDC3L and TLE6.

Translocation after co-transfection of human SCMC proteins

To further validate the physical interactions between human OOEP, KHDC3L, TLE6 and NLRP5, we examined whether one of these proteins could change the localization of the others when coexpressed in HeLa cells that are larger in size and are easier to be distinguished for their nucleus and cytoplasm. We first tagged the SCMC proteins with eGFP and separately transfected them into HeLa cells. Full-length and truncated TLE6 showed primarily cytoplasmic localization, whereas KHDC3L, NLRP5 and OOEP spread through both the nucleus and cytoplasm (Fig. 6A and D and Supplementary data, Table S2). None of proteins had a specific nuclear localization. To translocate the Cherry-labeled SCMC proteins into the cell nucleus, we tagged NLRP5 and KHDC3L proteins with an N-terminal nuclear localization signal (NLS). Most of NLRP5-pmCherry-NLS or KHDC3L-pmCherry-NLS was localized in the nucleus (Fig. 6B and C and s). Considering no interaction between truncated TLE6 (a negative control) and other components of the human SCMC proved by co-IP

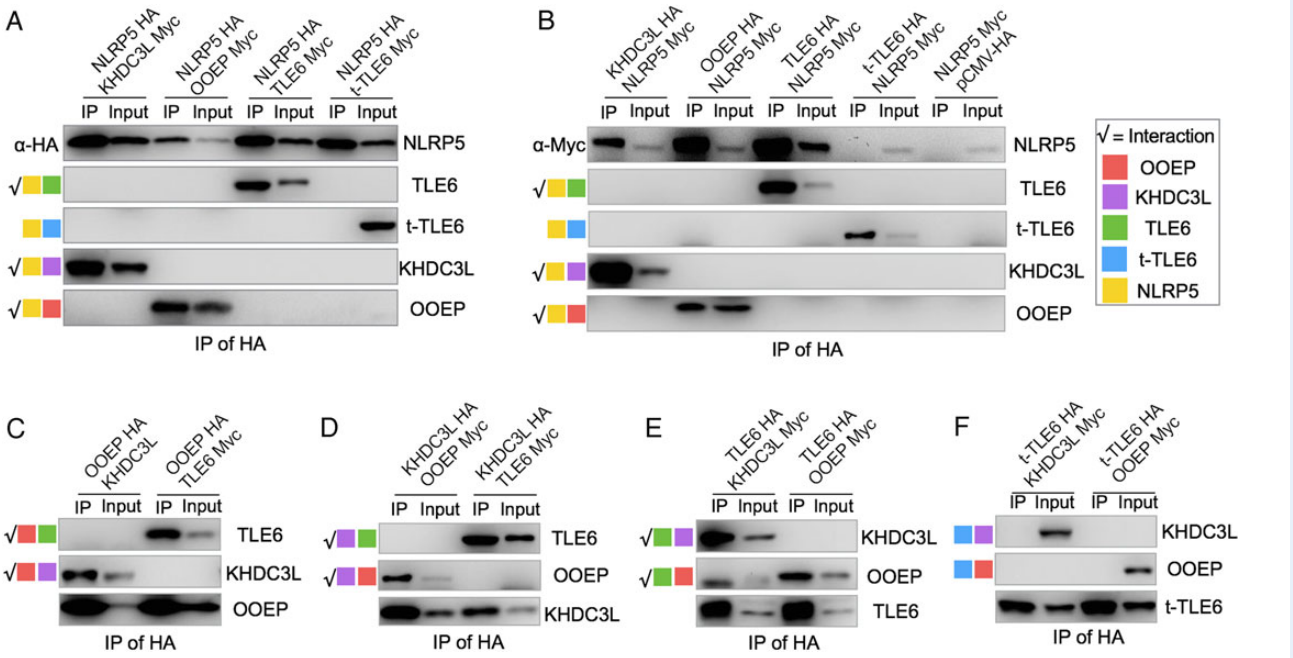
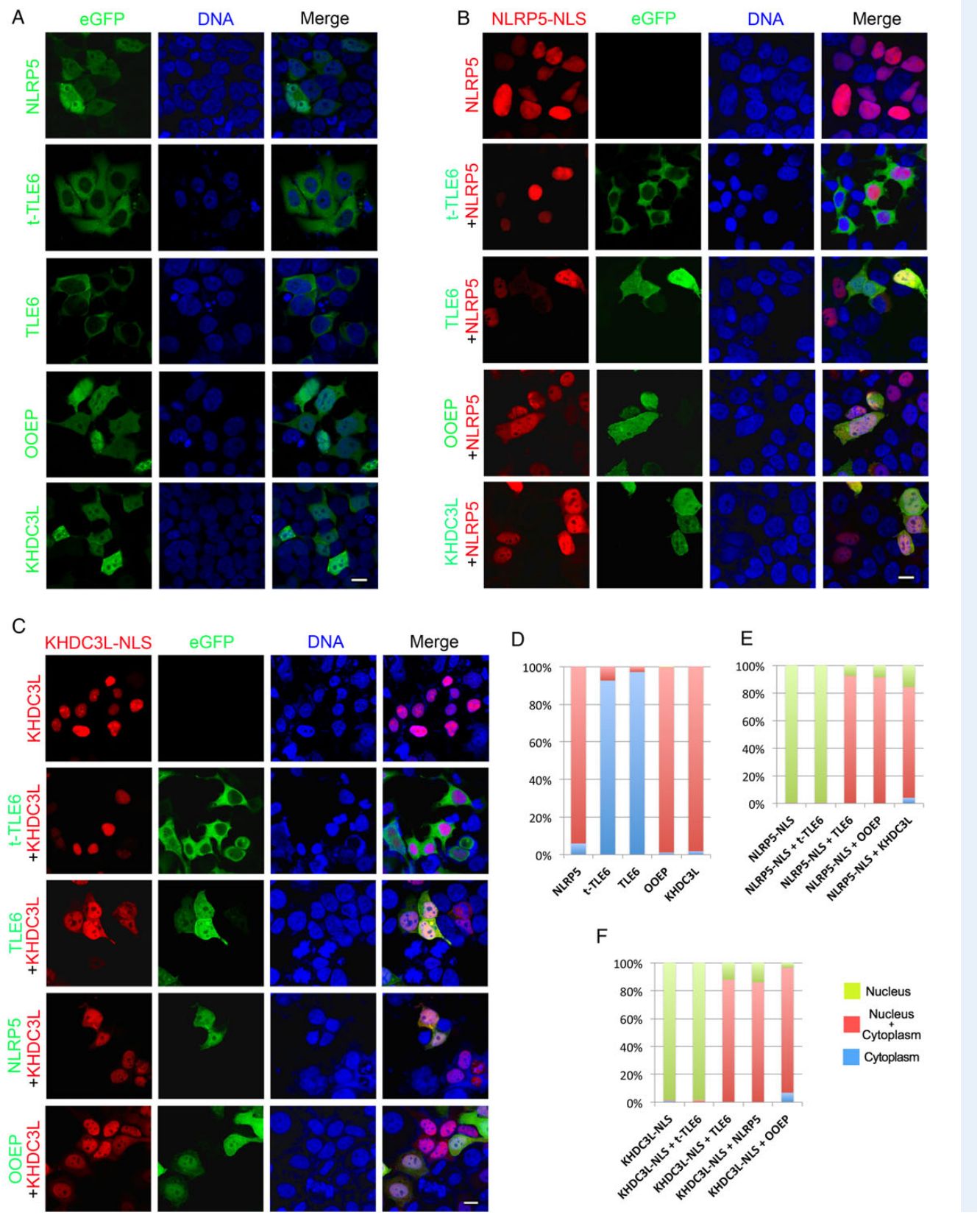


Figure 5 Interactions among the SCMC proteins examined by Co-IP. (**A**) NLRP5-HA was co-transfected into HEK293T cells with FILIA-Myc, OOEP-Myc, full-length (TLE6) or truncated TLE6-Myc (t-TLE6). Anti-HA was used to precipitate NLRP5 and associated proteins. Immunoblots were probed with antibodies to Myc and HA to detect the pull-down proteins (TLE6, OOEP, KHDC3L) and NLRP5-HA (positive controls), respectively. (**B**) NLRP5-Myc was co-transfected into HEK293T cells with KHDC3L-HA, OOEP-HA, full-length or truncated TLE6-HA, pCMV-HA (vector only negative control). Anti-HA was used to precipitate KHDC3L, OOEP, TLE6 and associated proteins. Immunoblots were probed with antibodies against Myc to detect NLRP5 and against HA as a positive control. (**C**) OOEP-HA was co-transfected into HEK293T cells with either KHDC3L-Myc or full-length TLE6-Myc. Anti-HA was used to precipitate OOEP and associated proteins. Immunoblots were probed with antibodies to HA and to Myc, to detect OOEP, KHDC3L and TLE6, respectively. (**D**) Same as in (C), except that KHDC3L-HA was co-transfected into HEK293T cells with either OOEP-Myc or TLE6-Myc. (**E**) Same as in (C), except that TLE6-HA was co-transfected into HEK293T cells with either KHDC3L-Myc or OOEP-Myc. (**F**) Same as in (C), except that truncated TLE6-HA was co-transfected into HEK293T cells with either KHDC3L-Myc or OOEP-Myc.



experiments (Fig. 5A, B and F), we co-transfected truncated TLE6-eGFP (t-TLE6) and NLRP5-pmCherry-NLS or KHDC3L-pmCherry-NLS into HeLa cells to examine if their localization would be changed by the co-transfection. Neither NLRP5-pmCherry-NLS nor KHDC3L-pmCherry-NLS was affected by truncated TLE6 on their localization, and vice versa, the cytoplasmic localization of truncated TLE6 was not affected as well (Fig. 6A, B, E, F, Supplementary data, Fig. S6–9 and Table S2). These results showed that the localizations of NLRP5-pmCherry-NLS or KHDC3L-pmCherry-NLS were not affected by the co-transfection with their no-binding protein.

Next, we co-transfected HeLa cells with the NLRP5-pmCherry-NLS or KHDC3L-pmCherry-NLS and other eGFP-tagged SCMC proteins. Compared with the single transfection in which most of NLRP5-pmCherry-NLS in the nucleus, over 80% of NLRP5-pmCherry-NLS located in the cytoplasm and nucleus after co-transfection with other SCMC proteins (Fig. 6B, E and Supplementary data, Table S2). Also, over 90% TLE6-eGFP, which originally located in cytoplasm, was observed to be located in the cytoplasm and nucleus after it was coexpressed with NLRP5-pmCherry-NLS (Fig. 6B, E and Supplementary data, Table S2). Similar results were obtained for the co-expression of KHDC3L-pmCherry-NLS with the other three proteins (Fig. 6C, F and Supplementary data, Table S2). Thus, the translocations of these proteins when they were coexpressed in cells further support the physical interactions between human SCMC proteins.

Discussion

Since it was discovered as a maternal effect gene in mouse, NLRP5 has been extensively studied as an oocyte- and early embryo-specific protein in other species, including *Homo sapiens*, with the results suggesting that it has a conserved function in mammals (Tong *et al.*, 2002; Pennetier *et al.*, 2004; McDaniel and Wu, 2009). FILIA was first identified as a binding partner of MATER (Ohsugi *et al.*, 2008) and was later characterized as a maternal effect gene in mouse (Zheng and Dean, 2009). Recently, KHDC3L was identified as one of the recessive genes responsible for FBHM (Parry *et al.*, 2011). In the present study, we characterized the expression of human *NLRP5*, *KHDC3L*, *OOEP* and *TLE6* by real-time RT-PCR and immunofluorescence staining. Our results show that human SCMC genes have similar expression patterns and that all of these genes are specifically enriched in oocytes and early embryos. We also demonstrate that all human SCMC proteins co-localize in the subcortex of early human embryos. In addition, we show that the human SCMC proteins physically interact when these genes are co-expressed in cell lines. Taken together, the similar expression patterns, the subcortical co-localization and the physical interactions of the four cognate proteins define a human SCMC. To the best of our knowledge, the SCMC is the first characterized maternal protein complex in *Homo sapiens*.

Maternal recessive effects have been known in *Drosophila* as early as 1980 (Nusslein-Volhard *et al.*, 1980) and have been widely studied in other animal models (Morisato and Anderson, 1995; Bowerman, 1998; Dosch *et al.*, 2004; Wagner *et al.*, 2004; Heasman, 2006). However, until recently, very few genes have been shown to be related to this phenomenon in mammals (Li *et al.*, 2010). Owing to technological limitations and the scarcity of biological material, maternal recessive effects remain largely unclear in *Homo sapiens*. Previously, only two genes (*NLRP7* and *KHDC3L*) have been shown to be associated with human maternal effect recessive mutation. *NLRP7* was identified as a recessive mutations in FBHM and was the first maternal effect gene identified in human (Murdoch *et al.*, 2006). Subsequent studies suggest that mutations of *NLRP7* are major causes of FBHM (Qian *et al.*, 2007; Kou *et al.*, 2008; Hayward *et al.*, 2009; Wang *et al.*, 2009; Nguyen and Slim, 2014). In searching for cases in which the FBHM defect was not associated with the *NLRP7* locus, *KHDC3L* was identified as the second maternal effect gene in human (Parry *et al.*, 2011). Currently, the roles of *NLRP7* and *KHDC3L* on the pathology of FRHM remain unclear. Although the phenotypes of mutations in mouse *Filia* and human *KHDC3L* described in the current literature are not completely identical (Zheng and Dean, 2009; Parry *et al.*, 2011), the maternal effect recessive phenomena and the orthologues in the same complex indicate the existence of similar functions and mechanisms of these genes in mouse and human early embryonic development. Thus, the mutations of the SCMC genes may result in human maternal recessive diseases.

Among infertile women seeking reproductive assistance from clinical IVF programs, not all reach a desirable outcome because of the failure of *in vitro* fertilized oocytes to develop to term due to unknown causes (Alikani *et al.*, 1999; Ostrowski *et al.*, 2001). However, KHDC3L and NLRP7 are the only maternal effect genes that have been identified in humans. The SCMC functions as a whole, and absence of any of its core components precludes the formation of the mouse SCMC and leads to embryo arrest at the 2-cell stage (Li *et al.*, 2008; Yu *et al.*, 2014). The mouse SCMC has a molecular weight between 669 and 2000 kDa, which is considerably in excess of the total mass (250 kDa) of the four identified proteins (Li *et al.*, 2008), implying the existence of other components. The absence of the core components of SCMC disrupts the complex that may result in the arrest of embryonic development at the preimplantation stage, which may be much earlier than clinical observations. Thus, some clinical undiagnosed infertile syndromes may be caused by the absence of the core (NLRP5, OOEP or TLE6) or other unidentified components of SCMC. Encouragingly, we observed

Figure 6 Translocation of the SCMC proteins after their co-transfection into cells. (**A**) eGFP-tagged NLRP5, truncated TLE6 (t-TLE6), full-length TLE6 (TLE6), OOEP and KHDC3L were, respectively, transfected into HeLa cells. After stained with Hoechst 33342, the cells were imaged with a Zeiss LSM 780 microscope. Scale bar, 10 μm. (**B**) NLRP5-NLS-pmCherry was transfected into HeLa cells and was observed in the nucleus. Translocation of NLRP5-NLS-pmCherry after co-transfection with TLE6-eGFP, OOEP-eGFP, KHDC3L-eGFP, but not t-TLE6-eGFP was observed using fluorescence microscopy. DNA was stained with Hoechst 33342. Scale bar, 10 μm. (**C**) KHDC3L-NLS-pmCherry was transfected into HeLa cells and was observed in the nucleus. However, KHDC3L-NLS-pmCherry was observed in both nucleus and cytoplasm after co-transfection with TLE6-eGFP, NLRP5-eGFP, OOEP-eGFP, but not t-TLE6-eGFP. DNA was stained with Hoechst 33342. Scale bar, 10 μm. (**D**) The histogram shows the percentage of subcellular localization of the five proteins in HeLa cells. (**E**) The percentage of the cells showing a particular localization of NLRP5-NLS-pmCherry transfected alone or with t-TLE6-eGFP, TLE6-eGFP, OOEP-eGFP or KHDC3L-eGFP. (**F**) The percentage of the cells with specific localizations of KHDC3L-NLS-pmCherry transfected alone or transfected together with t-TLE6-eGFP, TLE6-eGFP, NLRP5-eGFP or OOEP-eGFP.

the mislocalization of NLRP5 in some oocytes and zygote derived from IVF failure; in these contexts, NLRP5 lost its subcortical localization and diffused in the cytoplasm, as observed from staining for OOEP, TLE6 and KHDC3L (data not shown). Confirming whether this is indeed the consequence of disruption of the SCMC in the oocyte and 1-cell embryos from IVF failure deserves further investigation. To this end, genome-wide mutation analyses such as genome-wide association study and single cell genome analysis of human oocytes (Hou *et al.*, 2013) may serve as powerful tools to identify potential maternal mutations leading to the dysfunction of the SCMC.

Supplementary material

Supplementary Material is available at *http://molehr.oxfordjournals.org/* online.

Acknowledgements

We appreciate the patients who donated oocytes, embryos and tissues, and we appreciate Dr Lawrence M. Nelson at National Institutes of Health for kindly providing the NLRP5-pRSETC plasmid and Dr Jurrien Dean for the critical review of the manuscript.

Authors' roles

K.Z. designed and performed the major experiments, analyzed the data and wrote the manuscript. L.Y. designed the experiments and analyzed the data. X.Z. and X.L. participated in the experiments of the cell lines. T.W. and J.Y. collected the human samples. X.L. analyzed the data. J.Q. and L.L. initiated, funded, organized and designed the study, analyzed the data and wrote the manuscript. All authors approved the final manuscript.

Funding

The work was supported by the National Basic Research Program of China (2011CB944501, 2012CB944401, 2012CB944404), the National Natural Science Foundation of China (31171382, 30971655, 31230047), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA01010103) and the Hundred Talents Program of Chinese Academy of Sciences.

Conflict of interest

The authors declare no competing financial interests.

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