

Maternally derived FILIA-MATER complex localizes asymmetrically in cleavage-stage mouse embryos

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Initial cell lineages that presage the inner cell mass and extra-embryonic trophectoderm are established when eight blastomeres compact to form polarized morulae in preimplantation mouse development. FILIA has been identified as a binding partner to MATER (maternal antigen that embryos require; also known as NLRP5), which is encoded by a maternal effect gene. Products of each gene are detected in growing oocytes and, although transcripts are degraded before fertilization, the cognate proteins persist in early blastocysts. The two proteins co-localize to the cytocortex of ovulated eggs, where the stability of FILIA is dependent on the presence of MATER. After fertilization, FILIA-MATER complexes become asymmetrically restricted in the apical cytocortex of two-cell embryos due to their absence in regions of cell-cell contact. This asymmetry is reversible upon disaggregation of blastomeres of the two- and four-cell embryo. Each protein persists in cells of the preimplantation embryo, but the continuous cell-cell contact of 'inner' cells of the morulae seemingly precludes formation of the subcortical FILIA-MATER complex and results in cell populations that are marked by its presence ('outer') or absence ('inner'). Thus, the FILIA-MATER complex provides a molecular marker of embryonic cell lineages, but it remains to be determined if the molecular asymmetry established after the first cell division plays a role in cell fate determinations in the early mouse embryo. If so, the plasticity of the FILIA-MATER complex localization may reflect the regulative nature of preimplantation mouse development.

KEY WORDS: Preimplantation mouse development, Maternal effect genes, FILIA-MATER complex, Apical cytocortical polarization in blastomeres

INTRODUCTION

Following fertilization, the mouse embryo undergoes three mitotic cell divisions before compacting at eight cells to form individually polarized cells. The formation of the blastocoele two cell divisions later in the 32-cell blastocyst establishes an inner cell mass (ICM) and mural trophectoderm at one end of an embryonic-abembryonic axis and polar trophectoderm at the other (Rossant and Tam, 2004). Cell-fate determination has been ascribed to cell position at the 8-16 cell stage with progeny of all 'outer' cells forming the trophectoderm and the progeny of the 'inner' cells preferentially forming the ICM. Differences between the 'inner' and 'outer' cells based on asymmetrical division of cytoplasmic factors might prompt differences in gene expression that would lead to subsequent developmental commitment (Johnson and McConnell, 2004). However, the molecular basis for establishing these differences has not been established and could reflect maternal and/or embryonic gene products.

Activation of the embryonic genome in mice begins late in the one-cell zygote and is fully underway by the two-cell cleavage stage (Flach et al., 1982). In simpler model organisms, there is compelling evidence that persistent gene products from the egg are required for successful embryogenesis and axes formation. However, in mice such effects have been documented only more recently and constitute a rapidly evolving area of investigation. 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 (Howell et al., 2001; Bourc'his et al., 2001; Leader et al., 2002; Ye et al., 2005) development. *Mater* (*Nlrp5* – Mouse Genome Informatics) was one of the earliest maternal effect genes molecularly characterized in mice (Tong et al., 2000b).

MATER is an oocyte-specific protein first identified as an antigen associated with a mouse model of autoimmune oophoritis, a T-cell mediated inflammatory disease of the ovary (Tong and Nelson, 1999). The 125 kDa protein, encoded by a single-copy gene on Chromosome 7 (Tong et al., 2000a), has been more recently identified as a NALP protein (Tschopp et al., 2003), the largest clade in the Caterpillar family (Harton et al., 2002). Its original name derives from the acronym 'Maternal Antigen That Embryos Require' based on the phenotype observed in genetically altered mice. *Mater*^{mi/mi} male mice are unaffected; females attain normal sexual maturity with intact ovarian folliculogenesis and the ability to ovulate eggs that can be fertilized. Although most embryos undergo the first division, early embryonic lethality leads to a sterile phenotype. The arrested development is observed only in embryos derived from homozygous mutant females and cannot be rescued by wild-type males (Tong et al., 2000b).

Although *Mater* gene expression is restricted to growing oocytes and transcripts are not observed in cleavage-stage embryos, MATER protein persists in early development to the blastocyst stage (Tong et al., 2004). 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 leucine-rich repeat (28-29 amino acids) near the carboxyl terminus (Tong et al., 2000a) is a motif implicated in protein-protein interactions (Kobe and Kajava, 2001). Using specific domains or the entire MATER protein as bait, physiologically relevant interacting proteins have not been detected in yeast two-hybrid screens (T.

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Schulz, personal communication). However, comparing protein profiles of eggs from wild-type and *Mater^{tm/tm}* mice has led to the identification of FILIA1 as a binding partner for MATER.

MATERIALS AND METHODS

Reagents

PBS (20 mM phosphate buffer, 150 mM NaCl, pH 7.4); PBS/PVP (PBS, 0.3% polyvinylpyrrolidone); PBS/Triton (PBS, 0.5% Triton X-100); PBS/FCS (PBS, 1% bovine fetal calf serum); PBS/BSA (PBS, 1% bovine serum albumin); Complete Mini, EDTA-free protease inhibitors (one tablet per 10 ml, Roche Diagnostics, Indianapolis, IN); collagenase/DNase (0.1% Type III collagenase, 0.02% DNase I, Worthington Biochemical, Lakewood, NJ); MOPS (3-morpholinopropanesulfonic acid, Ambion, Austin, TX); Protein-A and protein-G Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ); High Range and Double Color Precise Molecular Markers (BioRad, Hercules, CA).

Isolation of ovaries, oocytes and embryos

Mater^{tm/tm} mice (Tong et al., 2000b) were obtained from Dr Lawrence Nelson, NICHD and others strains came from commercial vendors. Ovaries were removed from 10- to 21-day-old B6D2F₁ (C57BL/6×DBA/2)_{F1}, *Mater^{tm/+}* or *Mater^{tm/tm}* mice and oocytes were isolated using 21 gauge needles in M2 medium (Specialty Media, Phillipsburg, NJ) with or without collagenase/DNase (Eppig, 1976) and washed into fresh M2 medium. Female mice (3 or 8 weeks old) were stimulated with gonadotrophins and ovulated eggs and embryos were collected before or after mating, respectively (Ohsugi et al., 1996). All experiments were conducted in compliance with the guidelines of the Animal Care and Use Committee of the National Institutes of Health under a Division of Intramural Research, NIDDK approved animal study protocol.

Northern blot

Ovarian RNA was isolated from 10-day-old mice and purified with RNA-Bee (Tel-Test, Friendswood, TX). Total RNA (0.6 µg) was separated by electrophoresis on a 1% agarose gel containing 3% formaldehyde gel as described (Derman et al., 1981), except 20 mM MOPS was used instead of borate buffer. RNA was transferred to a Nytran N membrane (Schleicher & Schuell Bioscience, Keene, NH) and *Filia* (1.6 kb), *Mater* (3.3 kb) and *Zp2* (0.5 kb, exons 1-5) cDNAs, labeled with [³²P]dCTP using Ready-To-Go DNA Labeling Beads (Amersham Biosciences, UK), were hybridized sequentially to the membrane (Rankin et al., 1996). Hybridization signals were obtained by autoradiography and quantified on a FLA-5000 Phosphoimager (FujiFilm Medical Systems, Stamford, CT). RNA Ladder (0.24-9.5 kb, Invitrogen, Carlsbad, CA) was used as molecular mass marker.

Antibodies

Synthetic HPLC-purified peptides from the N-terminus of FILIA (MASLKRQTLVPLDHLKQGT) or the C-terminus of MATER (VIDGDWYASDEDDRNWWKN) were conjugated to KLH and used to immunize sheep and rabbits, respectively. Antisera were affinity-purified with the cognate peptide (Bethyl Laboratories, Montgomery, TX and Spring Valley Laboratories, Woodbine, MD, respectively), and secondary antibodies for immunofluorescence and immunoblots were obtained from Invitrogen, unless otherwise indicated.

Immunoblotting

Oocytes or embryos were washed with PBS/PVP, lysed in loading buffer (20 µl) and analyzed by SDS-PAGE (Laemmli, 1970) on 4-20% gradient gel (Invitrogen). Immunoblots were performed (Ohsugi et al., 1996) using antibodies to FILIA (1 µg/ml) or MATER (0.5 µg/ml) as primary and horseradish peroxidase (HRP)-conjugated anti-sheep or anti-rabbit antibody, respectively, as secondary antibodies. Images were obtained with ECL Plus (Amersham Pharmacia Biotech) and quantified on a FLA-3000 Luminescent Image Analyzer (FujiFilm Medical Systems).

Two-dimensional gel electrophoresis

Embryos (30) derived from wild-type or *Mater^{tm/tm}* females were incubated (3 hours) in 100 µl of M16 medium (Specialty Media) containing 2 µl of 10 µCi/µl [³⁵S]methionine/[³⁵S]cysteine (PerkinElmer, Boston, MA). After rinsing in PBS/PVP, embryos were lysed with 20 µl of 9 M urea, 4%

CHAPS, 40 mM Tris, 20 mM dithiothreitol (DTT) and stored at -80°C. The lysates were added to rehydration solution (105 µl) containing IPG buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer, pH 4-7, Bromphenol Blue, 20 mM DTT). Proteins were first separated by overnight isoelectric focusing (50 V, 12 hours; 500 V, 1 hour; 1000 V, 1 hour; 8000 V, 3 hours) using a 7 cm Immobiline DryStrip, pH 4-7 on an IPGphor Isoelectric Focusing Unit (Amersham Pharmacia Biotech) and then by SDS-PAGE using an 8% polyacrylamide gel (Invitrogen). The gels were fixed with 10% acetic acid (15 minutes), incubated with 1 M sodium salicylate (15 minutes) and visualized by fluorography (Bonner and Laskey, 1974). Positive signals were rendered red in wild-type embryos or green in embryos derived from *Mater^{tm/tm}* eggs and merged in Adobe Photoshop.

Mass spectrometry

Approximately 2000 eggs from wild-type or *Mater^{tm/tm}* mice were analyzed by SDS-PAGE using 8% polyacrylamide gels (Laemmli, 1970) and protein bands were visualized with Simply Blue Safe Stain (Invitrogen). Bands judged decreased in *Mater^{tm/tm}* were excised from the lane containing proteins from wild-type eggs, digested with trypsin and peptides were identified by microscale LC-MS/MS at the NIDDK Proteomics and Mass Spectrometry Facility.

RT-PCR

Total RNA from 3-week-old mouse kidney, ovary, uterus, testes, brain, heart, lung, liver, spleen, stomach, small intestine and muscle was purified with RNeasy Mini Kit (Qiagen, Valencia, CA) and ~50 ng aliquots were used for individual RT-PCR reactions. Forward and reverse oligonucleotide primers for *Filia* (5'-TAGGCTTCCTGCGGTGAAA-3'; 5'-TGAGCCAGATCAG-TGAGCAT-3') and β-actin (Nichols et al., 1998) were used in a OneStep RT-PCR Kit (Qiagen) under the following conditions: 50°C (30 minutes); 95°C (15 minutes); 35 cycles of 94°C (30 seconds), 55°C (30 seconds), 72°C (30 seconds); 72°C (10 minutes). PCR products were separated by 1.2% agarose gel electrophoresis and visualized after staining with ethidium bromide. The primers for *Filia* recognized both isoforms.

In situ hybridization

Ovaries from 2-week-old mice were fixed (2 hours, RT) in Histochoice MB Fixative (Electron Microscopy Sciences, Hatfield, PA) and washed with 50% and 70% ethanol before sectioning (American Histolabs, Gaithersburg, MD). GeneSTAR Sense (5'-GCAACCTGGTGGGCTCCCTGAGGTGTG-CTGGGCAAAGGCTTCATCA-3') and antisense, 48-mer DIG-labeled oligonucleotide probes were designed and synthesized by GeneDetect (<http://www.genedetect.com>). After deparaffinizing and rehydration, ovarian sections were permeabilized with proteinase K, hybridized (37°C, ON) according to the manufacturer's instructions (GeneDetect, Bradenton, FL). Hybridization signals were detected with tyramide signal amplification (TSA) and developed with diaminobenzidine tetrahydrochloride (DAB) according to the manufacturer's instructions (Dako, Carpinteria, CA). Tissues were counterstained with hematoxylin before mounting and imaging on an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY).

In vitro translation

Ovarian poly (A)⁺ RNA was purified from 10 ovaries (10-day-old mice) with Dynabeads (Dyna, Oslo, Norway) and full-length cDNA was prepared with a cDNA Amplification Kit (Clontech, Mountain View, CA). *Filia* cDNA was then amplified with a half concentration of BD Advantage GC 2 Polymerase Mix (Clontech) using Adaptor Primer 2 and 5'-AAGGT-CAGCGATGCTGCCACGCAGT-3' as a reverse primer for 5' RACE-PCR or Adaptor Primer 2 and 5'-AACGAGGCTGCCACCGAGCAGGCTT-3' as a forward primer for 3' RACE-PCR. The overlapping PCR products were cloned into pBS SK⁺ plasmid to generate full-length *Filia* cDNA, the sequence of which was confirmed by capillary dideoxy DNA sequencing (MWG Biotech, High Point, NC). Two sequences were obtained; one was 1.6 kb and the other 1.2 kb. The longer isoform contained an additional 0.4 kb internal sequence. *Filia* 1.6 and *Filia* 1.2 cDNA in pBS SK⁺ plasmid were independently translated into protein with a TNT Coupled Wheat Germ Extract System (Promega, Madison, WI) utilizing [³⁵S]methionine. The gene products were separated by SDS-PAGE and visualized with fluorography as described above.

Quantitative real-time RT-PCR

Taqman probes and primers were obtained (Applied Biosystems, Foster City, CA) from existing stocks for MATER (Mm00488691_m1) and by custom design for the two FILIA isoforms (Fig. 4B) (FILIA 1.2: forward primer, 5'-GGGAAGGTCAGCGATGCT-3', reverse primer 5'-GGGA-AACAAAAACAGCTCTACTCTGT-3', probe 5'-CCCGATTCCTGCA-CCGAA-3'-MGB; FILIA 1.6 forward primer, 5'-GGCAGTCTCCCA-TTGAAGTCT-3', reverse primer 5'-GGGACAAGGCCCTAGAGACA-3', probe 5'-CCGGGCAGCATTCTCTAGA-3'-MGB. The specificity of the probes was confirmed by high-resolution gel electrophoresis as well as the plasmid controls in qRT-PCR and their efficiencies were determined using 10-dilutions of plasmid DNA as template (Pfaffl, 2001). Total RNA and ssDNA templates were obtained as described above and three independently obtained biological samples from each developmental time point were assayed using TaqMan Universal PCR Master Mix according to the manufacturer's protocol.

Whole-mount immunofluorescence

Isolated oocytes or embryos were washed (PBS/PVP), fixed (PBS, 2.0% paraformaldehyde, 45 minutes) and permeabilized in PBS, 0.2% Triton X-100, 5% BSA. After blocking (PBS/FCS, 1 hour), samples were incubated (1 hours, RT) with antibodies to FILIA (10 µg/ml, PBS/BSA) or MATER (0.6 µg/ml, PBS/BSA) and then visualized with Alexa 633-conjugated donkey IgG specific to sheep-IgG or Alexa 488-conjugated goat IgG specific to rabbit-IgG (1:200 in PBS/BSA). Nuclei were stained with Hoechst 33342 (10 µg/ml). Oocytes and embryos were imaged with an LSM 510 confocal microscope (Carl Zeiss) equipped with differential interference contrast optics (Rankin et al., 1999). The Alexa 488 fluorochrome was excited with a 488 nm Argon laser and emissions were detected through a BP500-550 nm filter. The Alexa 633 fluorochrome was excited with a 633 HeNe laser and emissions were detected through a BP650-710 nm filter.

Immunoprecipitation

Ovulated eggs were lysed (>20 minutes, on ice) in 400 µl PBS/Triton and Complete Mini, EDTA-free protease inhibitors. After centrifugation (13,000 rpm, 5 minutes), the supernatant was pre-cleared with pre-immune serum (2 µl) and Protein-A Sepharose (2 µl). Anti-FILIA antibodies (10 µl, 1 mg/ml) and Protein-G Sepharose (30 µl) were added contemporaneously and incubated (4°C, 2 hours). The immunocomplex with Protein-G Sepharose was pelleted and washed with PBS/Triton five times before SDS-PAGE and immunoblot with anti-MATER antibody as described above.

Expression of epitope-tagged MATER and FILIA

Expression vectors were constructed by subcloning cDNAs encoding near full-length MATER (amino acids 23-1111) or the 1.2 kb *Filia* isoform (amino acids 1-346) in pCMV-Myc and pCMV-HA (Clontech), respectively. 293T-cells (60 mm dish, ~80% of confluent) were co-transfected with DNA plasmids (3 µg each) using Lipofectamine and Plus Reagent according to the manufacturer's instructions (Invitrogen). After 48 hours, the cells were washed (2 ml, cold PBS), lysed in PBS/Triton with protease inhibitors, vortexed and incubated on ice (≥20 minutes). Particulate matter was removed by centrifugation (13,000 rpm, 5 minutes and then 10 minutes) and the supernatants used for immunoprecipitation. Lysates (5-50 µl) were diluted into 0.7-1.0 ml of 1% BSA in PBS/Triton plus protease inhibitors and incubated with Protein-A Sepharose (30 µl of 50% solution, 1 hour, 4°C). After centrifugation, the supernatant was transferred into new tubes before adding antibody and a 50% solution of Protein-A or -G Sepharose (30 µl) and incubating (ON, 4°C). Immunoprecipitates were collected by centrifugation (2000 rpm, 1 minute) and pellets were washed with PBS/Triton containing 1% BSA (2×) and PBS/Triton alone (3×). Sample buffer was added and the protein samples were separated by SDS-PAGE and detected by immunoblot. Anti-Myc antibodies were obtained from Clontech (Mountain Valley, CA) and Sheep TrueBlot (eBioscience, San Diego, CA) was used as the secondary antibody to avoid detection of denatured IgG bands.

Disaggregation of cleavage-stage embryos

Zonae pellucidae were removed from two-cell embryos or morulae by short incubation (20-30 seconds) in acidic Tyrode's solution (Chemicon International, Temecula, CA). Zona-free embryos were incubated for 2

hours in M16 and then 15-30 minutes in a Brinster's Ca²⁺/Mg²⁺-free medium (KD Medical, Columbia MD) with 0.3% PVP at 37°C. Embryos were disaggregated individually by gentle pipetting through the 50 µm tip (Stripper PGDTips, MidAtlantic Diagnostic, Mount Laurel, NJ) in 50 µl drops of M16 under oil. Inner cell masses were obtained from 3.5 day blastocysts by immunosurgery (Solter and Knowles, 1975) using rabbit anti-mouse antisera and guinea pig complement (SigmaAldrich, St Louis, MO). Within 2 hours after antibody addition, cells were fixed in 2% paraformaldehyde with 0.3% PVP and processed for detection of FILIA and MATER as described above. F-actin was detected by staining with phalloidin tetramethylrhodamine (50 µg/ml, Sigma-Aldrich).

RESULTS

mRNA and protein in *Mater^{tm/tm}* mice

MATER is an oocyte-specific protein encoded by a single copy gene in the mouse genome (Tong et al., 2000a). Using homologous recombination in embryonic stem cells, the *Mater* locus has been mutated by the insertion of a PGK-neomycin cassette in the second intron, but without invading adjacent exons. Although greatly diminished compared with wild-type or heterozygous eggs, mRNA encoding MATER can be detected in *Mater^{tm/tm}* mice (Fig. 1A), as can residual amounts of protein (Fig. 1B). Thus, although initially reported as a null mutation, the insertion of the neomycin cassette in the second intron appears to destabilize the *Mater* transcript and cause a severe hypomorph phenotype that affects early mouse development (Tong et al., 2000b).

To assess the biochemical sequelae of MATER loss on early development, two-dimensional gels were used to examine de novo protein synthesis in one- and two-cell embryos derived from wild-type and *Mater^{tm/tm}* mice (Fig. 1C). Newly synthesized proteins were labeled with [³⁵S]methionine/[³⁵S]cysteine and, after separation by gel electrophoresis and identification by fluorography, faux labeled red or green, respectively. At the one-cell stage, most of the proteins present in wild type were also present in mutant mice, as evidenced by the co-localization (yellow) in the merged images (Fig. 1Ca-c). At the two-cell stage there was a dramatic decrease in de novo protein synthesis in embryos derived from *Mater^{tm/tm}* mice compared with wild type, and relatively few of the proteins present in wild-type embryos were observed in *Mater^{tm/tm}*-derived embryos (Fig. 1Cd-f).

Identification and expression of *Filia*

To determine if early embryonic differences arose during oogenesis, ovulated eggs (~2000) from wild-type or *Mater^{tm/tm}* mice were analyzed by SDS-PAGE gel electrophoresis, and the abundance of proteins was compared after staining with Simply Blue Safe Stain (Fig. 2A). In addition to the loss of MATER (125 kDa), there was also a dramatic decrease in the abundance of a protein (~50 kDa), which was designated FILIA (FILIA and MATER are Latin for daughter and mother, respectively). After excision and digestion with trypsin, the protein was identified by mass spectrometry as a hypothetical protein of unknown function encoded by Riken cDNA 2410004A20.

Using RT-PCR and primers based on the cDNA sequence, *Filia* transcripts were detected in ovary but not testes or somatic tissues, using actin as a control of RNA integrity (Fig. 2B). To examine the specificity of expression within the ovary, Histochoice MB-fixed, paraffin sections from 2-week-old mice were hybridized with antisense and sense (control) probes (Fig. 2C). *Filia* transcripts were readily detected in growing oocytes with only background hybridization in the surrounding somatic tissue. *Filia* is a single copy gene with three exons located on mouse chromosome 9qD. Two isoforms (1.6 and 1.2 kb) were detected by northern blot analysis of

Fig. 1. Expression of *Mater* in mice with targeted mutation.

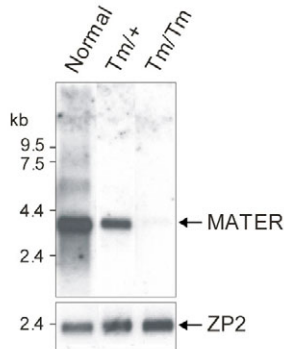
(A) Northern blot of total RNA (0.6 μ g) from 10-day-old ovaries isolated from wild-type, *Mater*^{tm/+} and *Mater*^{tm/tm} mice. After separation by gel electrophoresis and transfer to nylon membrane, blots were hybridized with ³²P-labeled *Mater* or *Zp2* (control) cDNA, washed and imaged.

(B) Immunoblot of ovulated eggs (10) isolated from wild-type, *Mater*^{tm/+} and *Mater*^{tm/tm} mice. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with peptide affinity-purified antibody to MATER and visualized by chemiluminescence.

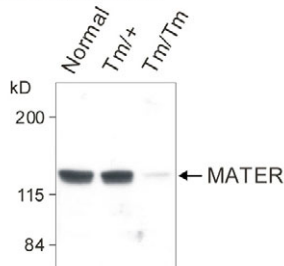
(C) Two-dimensional gels of de novo synthesized proteins in one-cell (a-c) and two-cell (d-f) mouse embryos, collected from gonadotrophin-stimulated, mated females 24 and 43 hours

after administration of hCG, respectively. After in vitro labeling with [³⁵S]methionine/[³⁵S]cysteine, 30 embryos were focused on isoelectric gels (pH 4-7) and separated by SDS-PAGE. After visualization with fluorography, radioactivity was rendered red (wild type, a,d) or green (*Mater*^{tm/tm}, b,e) and merged (c,f). Size (kb) is indicated on the left (A) and molecular masses (kDa) are indicated on the left (B) or right (C).

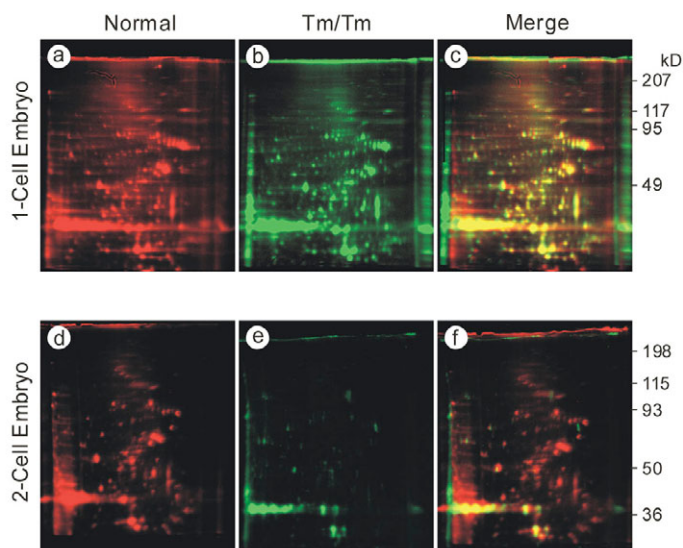
A. Northern Blot



B. Western Blot



C. Two-dimensional Protein Gels



ovarian RNA isolated from wild-type, *Mater*^{tm/+} and *Mater*^{tm/tm} mice (Fig. 2D), and each is present in the mouse EST database. The 1.2 kb isoform was more (~fivefold) abundant than the 1.6 kb isoform, and the quantity of neither was perturbed in *Mater*^{tm/tm} mice. However, although the near absence of *Mater* mRNA did not affect *Filia* mRNA, the absence of the MATER protein had a profound affect on the abundance of FILIA protein (Fig. 2A).

Distinguishing between the two isoforms

The single copy *Filia* gene is transcribed as two isoforms, *Filia* 1.6 and *Filia* 1.2, that potentially encode proteins with 440 (49,995 Da) and 346 (37,995 Da) amino acids, respectively (Fig. 3A). Peptides detected by mass spectroscopy covered 45% of the smaller protein encoded by FILIA 1.2, and no peptides were detected from the 100 amino acids at the carboxyl-terminal of the conceptual protein uniquely encoded by *Filia* 1.6 (Fig. 4A). Analysis of FILIA protein by Dotplot (Wisconsin Sequence Analysis Package) and RADAR (Heger and Holm, 2000) demonstrated the presence of a 10-tandem, 23 amino acid repeat (Fig. 4B) near the carboxyl terminus of each isoform (amino acids 120-350 and 120-340, respectively; dotted line, Fig. 4A). Using either the amino acid or the encoding nucleic acid sequence to search the mouse genome, other proteins containing the repeat were not identified.

cDNA encoding each of the isoforms was transcribed and translated into protein in the presence of [³⁵S]methionine (Fig. 4C). The larger 1.6 kb isoform expressed a protein with an apparent molecular mass of 70 kDa on SDS-PAGE, significantly larger than that predicted by its amino acid composition (48 kDa). The smaller (1.2 kb) isoform expressed a protein with an apparent molecular mass of 50 kDa (also significantly larger than its predicted molecular mass, 38 kDa) and corresponded to the diminished band observed in oocytes isolated from *Mater*^{tm/tm} mice (Fig. 2A). The N-terminus

peptide (amino acids 1-20) common to both forms of FILIA was coupled to KLH and used to raise antisera in sheep for subsequent experiments.

Developmental expression of *Filia* and *Mater*

Using Taqman probes and quantitative real-time RT-PCR (qRT-PCR), the relative accumulation of transcripts encoding FILIA and MATER was determined during oogenesis and preimplantation development. *Filia* and *Mater* are each single-copy genes found on mouse Chromosomes 9 and 7, respectively, and primer sets were designed for *Filia* 1.6, *Filia* 1.2 (Fig. 3B) and *Mater* transcripts. The efficiency of each primer set was assayed over four orders of magnitude using plasmid DNA as substrate (Fig. 5A, inset). Each primer had a regression coefficient >0.999 and the relative efficiencies of the *Filia* 1.6, *Filia* 1.2 and *Mater* primer sets were 1.600, 1.904 and 1.861, respectively (Pfaffl, 2001).

mRNA was isolated from oocytes (50 μ m, 75 μ m), eggs and preimplantation embryos (two-cell, morula, blastocyst), and single-stranded cDNA was prepared from three independently obtained biological samples. Each sample was run in triplicate, normalized for the number of oocytes or embryos and corrected for the relative efficiencies of their primer sets (Pfaffl, 2001). The amount of *Filia* 1.2 in 50 μ m oocytes was set at 1.0 and the relative amounts of all three transcripts were determined during oogenesis and early development (Fig. 5A). The abundance of *Filia* 1.2 and *Mater* transcripts was comparable in growing oocytes, but *Filia* 1.6 was ~20% as abundant as *Filia* 1.2 and appeared to diminish in fully grown oocytes. As oocytes became transcriptionally quiescent during meiotic maturation and ovulation, all three transcripts virtually disappeared. However, unlike *Mater*, which remained absent in preimplantation embryos, FILIA (*Filia* 1.6>*Filia* 1.2) transcripts were detected in morula and early blastocysts.

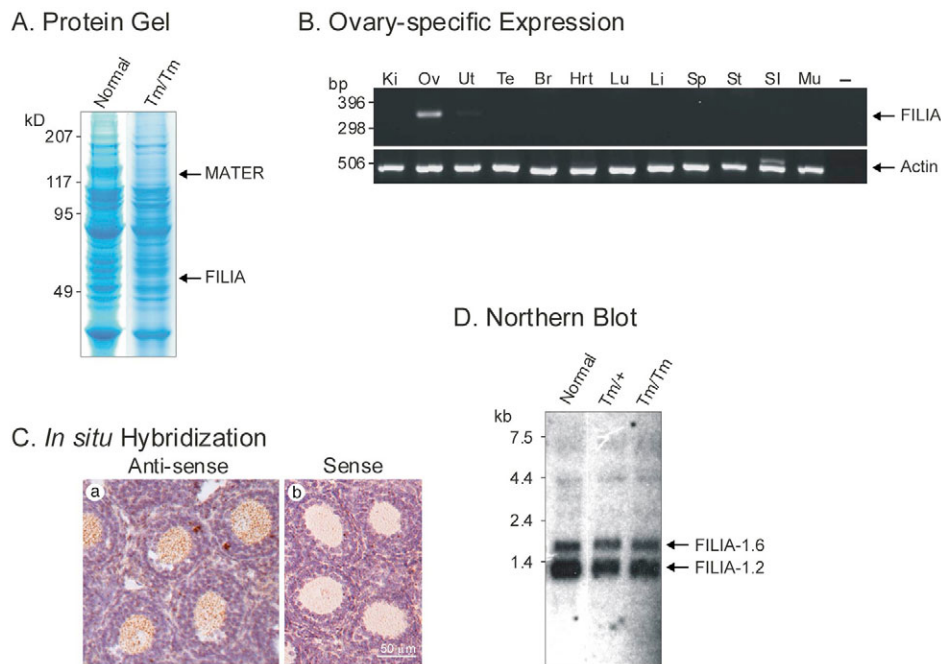


Fig. 2. Identification of FILIA. (A) SDS-PAGE of zona-free ovulated eggs (~2000) isolated from wild-type or *Mater^{tm/tm}* mice. After fixation and staining, a protein (~50 kDa) diminished in *Mater^{tm/tm}* eggs was designated FILIA and excised from the gel for analysis by mass spectrometry. Molecular masses (kDa) are indicated on the left. (B) RT-PCR of total RNA isolated from kidney, ovary, uterus, testis, brain, heart, lung, liver, spleen, stomach, small intestines, muscle and water using primers specific for *Filia* (top) or actin (bottom) to control for RNA integrity. Size (bp) is indicated on the left. (C) In situ hybridization of fixed, paraffin-embedded wild-type ovarian (2-week-old) sections probed with DIG-labeled antisense (a) and sense (b) synthetic *Filia* oligonucleotide probes. (D) Total RNA (0.6 μg) was isolated from ovaries of wild-type, *Mater^{tm/+}* and *Mater^{tm/tm}* mice, separated by electrophoresis, transferred to a nylon membrane and probed with ³²P-labeled FILIA cDNA. Molecular masses (kb) are indicated on the right. Scale bar: 50 μm. -, water; Br, brain; Hrt, heart; Ki, kidney; Li, liver; Lu, lung; Mu, muscle; Ov, ovary; Si, small intestines; Sp, spleen; St, stomach; Te, testis; Ut, uterus.

To complement the developmental profiles of transcript accumulation, oocytes, eggs and embryos (ten each) were assayed by immunoblots for FILIA and MATER proteins (Fig. 5B). In addition to antisera that recognized the N-terminus of both FILIA isoforms, a second antisera specific to MATER was produced in rabbits immunized with a carboxyl-terminus peptide (amino acids 1093-1111) coupled to KLH. This antisera was also peptide-affinity-purified and the resultant monospecific antibody had specificity similar to an earlier report (Tong et al., 2004). The smaller FILIA isoform (~50 kDa) and MATER (~125 kDa) proteins were detected in comparable amounts in growing oocytes, ovulated eggs and preimplantation embryos up to the morula stage of development (Fig. 5B). Although present at the blastocyst stage (E3.5), the abundance of each protein, as detected by immunoblot, was markedly decreased (Fig. 5B).

The predicted larger FILIA protein isoform (~70 kDa) was not detected in oocytes, eggs or preimplantation embryos using monospecific antibodies to the N-terminal peptide (20 amino acids) common to both FILIA isoforms, even though 1.6 *Filia* transcripts were present. Additional attempts to detect peptides unique to the 440 amino acid (~70 kDa) FILIA isoform in oocytes and embryonic stem cells after SDS-PAGE purification and microscale LC-MS/MS were not successful.

FILIA interacts with MATER

Growing oocytes (40 μm), eggs and early embryos were isolated, permeabilized and stained with monospecific antibodies to FILIA and to MATER. Each antibody was raised in a different species and

did not cross-react with the other target protein. FILIA and MATER were imaged by confocal microscopy using Alexa 633-conjugated anti-sheep antibody (green) specific to anti-FILIA antibodies and Alexa 488-conjugated anti-rabbit antibody (red) specific to anti-MATER antibodies (Fig. 6). Merging the two images demonstrated co-localization of the two proteins in the subcortex during oogenesis and in ovulated eggs. The peripheral localization of the FILIA and MATER persisted in cleavage-stage embryos and the protein complex was not detected from the 'inner' cells of morulae and the ICM of the early blastocysts (Fig. 6).

To determine if the two proteins physically interact, the monospecific antibodies to FILIA were used to immunoprecipitate whole cell lysates from ovulated eggs. The immunoprecipitated material was separated by SDS-PAGE and analyzed by immunoblot using antibodies specific to MATER (Fig. 7A). MATER was detected as a 125 kDa protein in egg lysate (Fig. 7A, lane 1) and was immunoprecipitated by anti-FILIA antibody from wild-type egg (Fig. 7A, lane 2), but not *Mater^{tm/tm}* egg lysates (Fig. 7A, lane 3). MATER was not immunoprecipitated with pre-immune antisera (Fig. 7A, lane 4). However, reciprocal experiments using monospecific antibodies to MATER to immunoprecipitate FILIA in whole cell lysates from ovulated eggs were not successful. Therefore, expression plasmids containing a Myc-tagged cDNA encoding near full-length MATER (amino acids 23-1111) or full-length HA-tagged FILIA (amino acids 1-346) were transiently co-transfected in heterologous 293T cells (Fig. 7B, lane 2). After immunoprecipitation with anti-Myc antibodies, FILIA that bound to full-length MATER was detected by immunoblot of cell lysates (Fig. 7B).

FILIA-MATER complex localization in early embryogenesis

After completion of the first cell division, the two blastomeres adhere to one another via calcium-dependent homotypic interactions of E-cadherin, a transmembrane adhesion protein (De Vries et al., 2004). Unlike the more uniform subcortical distribution in the egg, the FILIA-MATER complex assumed an apical localization in the two-cell embryo with specific exclusion in the region of cell-cell contact (Fig. 8Aa,b). By contrast, F-actin detected with phalloidin was present throughout the subcortex (Fig. 8Ac). In the absence of a zona pellucida, the removal of calcium from the culture media caused blastomeres to disassociate, and the FILIA-MATER complex quickly re-equilibrated to the same uniform, subcortical localization observed in the eggs and co-localized with F-actin, which remained unchanged (Fig. 8Ae-g). A similar re-localization of the FILIA-MATER complex after disaggregation of blastomeres was observed at the four-cell stage. This redistribution was reversible upon reaggregation of blastomeres in the presence of calcium or absorption of individual blastomeres to collagen-coated glass slides, where FILIA-MATER complex was excluded from the regions of contact (data not shown).

The continued exclusion of the FILIA-MATER complex from basolateral contacts as embryos divide led to differential detection among blastomeres. At the eight-cell stage, mitotic cell division parallel to the plane of polarization forms two daughter cells, each with the FILIA-MATER complex at the apical cytocortex. However, cell divisions orthogonal to the plane of polarization

(Johnson and Ziomek, 1981b; Sutherland et al., 1990) resulted in differentially endowed daughter cells with the parental FILIA-MATER complex detected at the apical subcortex of 'outer' but not 'inner' cells (Fig. 6, Fig. 8C). As noted at the two-cell stage, the asymmetric apical localization of the FILIA-MATER complex was lost when individual 'outer' cells were disaggregated from the compacted morula, but was maintained in clumps of cells that remained adherent to one another (Fig. 8B). Thus, the ability of the apical subcortical FILIA-MATER complex to redistribute into a symmetrical localization, first observed upon disaggregation of two-cell embryos, persists in the 'outer' cells of the compacted morula.

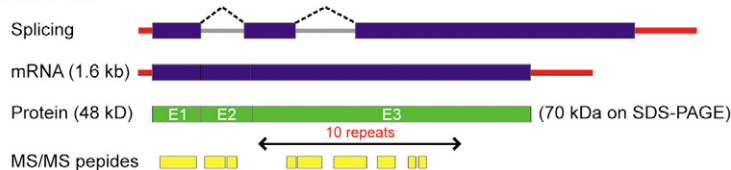
However, it was unclear whether the apparent absence of the FILIA-MATER complex from the subcortex of 'inner' cells reflected loss of protein or disaggregation of the complex due to continuous cell-cell contacts. Therefore, regions free of cell-cell contact were reestablished in 'inner' cells by immunosurgery in which 3.5 day blastocysts were flushed from the oviduct and treated with rabbit anti-mouse antisera and guinea pig complement to lyse 'outer' cells and recover the ICM (Solter and Knowles, 1975). The ICMs were then fixed, permeabilized and stained with antibodies to FILIA and MATER (Fig. 8D). As before (Fig. 6), the FILIA-MATER complex was present in the trophectoderm and was not visualized in the ICM of control blastocysts (data not shown). Following immunosurgery, the FILIA-MATER complex reassembled in the subcortex of the post-surgical ICM. Thus, the individual proteins persist in the

A. FILIA Isoforms

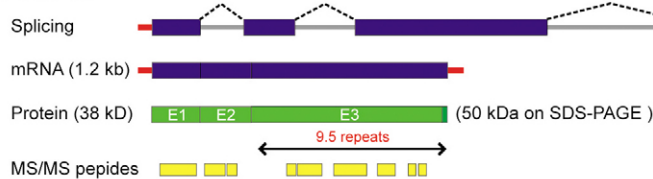
a. *Filia* (mouse Chr 9)



b. FILIA-1.6



c. FILIA-1.2



B. Real-time RT-PCR

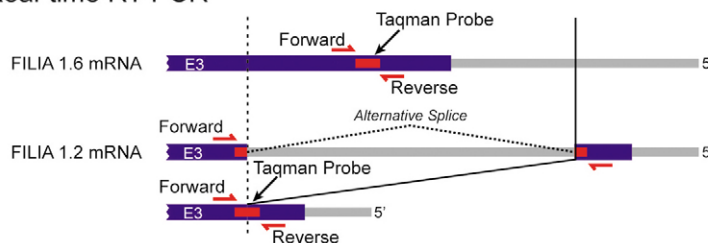


Fig. 3. Alternative splicing of *Filia* in mice.

(A) (a) *Filia* is a single-copy gene with three exons on chromosome 9 (73,235,088-73,237,040) that is transcribed as two isoforms. (b) The less abundant 1.6 kb transcript encodes a 440 amino acid protein with a smaller molecular mass (48 kDa) than predicted (70 kDa) by its mobility on SDS-PAGE. The tenfold repeat is indicated by double-arrow line, and peptides obtained by mass spectrometry (yellow rectangles) are aligned at the bottom. (c) Same as b for the 1.2 kb transcript that encodes a 346 amino acid protein with a smaller molecular mass (38 kDa) than predicted (50 kDa) by its mobility on SDS-PAGE. (B) Synthetic oligonucleotide primers and Taqman probes designed to distinguish the alternatively spliced 1.6 kb (upper) and 1.2 kb (lower) isoforms of *Filia* transcripts. E, exon.

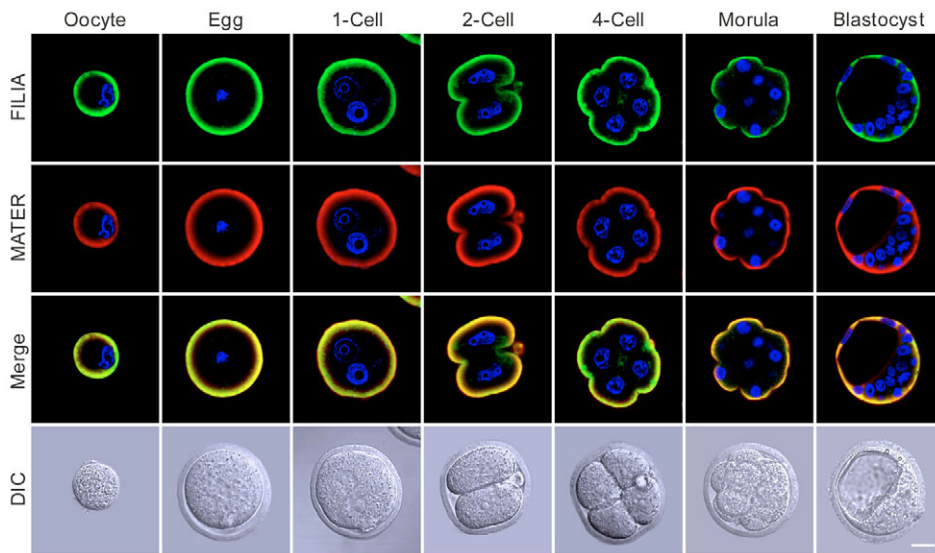


Fig. 6. Co-localization of FILIA and MATER by confocal microscopy.

Mouse oocytes, eggs, one-cell, two-cell and four-cell embryos, morulae and blastocysts (E3.5) were fixed, permeabilized and stained with peptide-purified antibodies to FILIA (green) and MATER (red). Co-localization of FILIA and MATER was determined by Merge (yellow) and oocytes/eggs/embryos were imaged by differential interference contrast (DIC) microscopy. Scale bar: 20 μm .

Early mouse development is regulative, and cells derived from blastomeres before compaction can participate in all tissues of the adult (Tarkowski, 1959; Kelly, 1977; Rossant, 1976; Papaioannou et al., 1989). Such plasticity does not seemingly preclude differences among embryonic cells during early cleavage stages (Rossant and Tam, 2004; Torres-Padilla et al., 2007), although there is controversy as to their effects on subsequent embryonic polarity (Hiiragi et al., 2006; Zernicka-Goetz, 2006; Kurotaki et al., 2007). During the first three cell divisions, embryonic blastomeres appear morphologically symmetric. However, as the next cell division initiates, the eight-cell embryo undergoes Ca^{2+} -mediated compaction, which polarizes individual cells (Ziomek and Johnson, 1980; Johnson and Ziomek, 1981a). Subsequent cell divisions orthogonal to the apical-basal axis of the polarized cell results in two distinct cell populations: 'inner' cells that form the embryonic ectoderm/endoderm ICM; and 'outer' cells that contribute progeny to the trophectoderm (Tarkowski and

Wroblewska, 1967; Johnson and Ziomek, 1981b; Sutherland et al., 1990). The establishment of these two cell fates involves homotypic interactions of maternal stores of E-cadherin along the basolateral membranes interacting with subcortical elements of the cytoskeleton (De Vries et al., 2004). In contrast to the flattening of these cell-cell contacts, the apical subcortical region remains rich in microvilli, forming a polar domain that is stable as preimplantation development progresses (Reeve and Ziomek, 1981; Johnson and Ziomek, 1981b). Thus, following compaction, the embryo becomes an epithelized sphere with distinct polarization of individual blastomeres and two cell populations (for reviews, see Muller, 2001; Johnson and McConnell, 2004).

FILIA and MATER proteins are synthesized during oogenesis, but not in cleavage-stage embryos that lack the cognate transcripts. However, the two maternally derived proteins persist during preimplantation development and are potential markers for non-

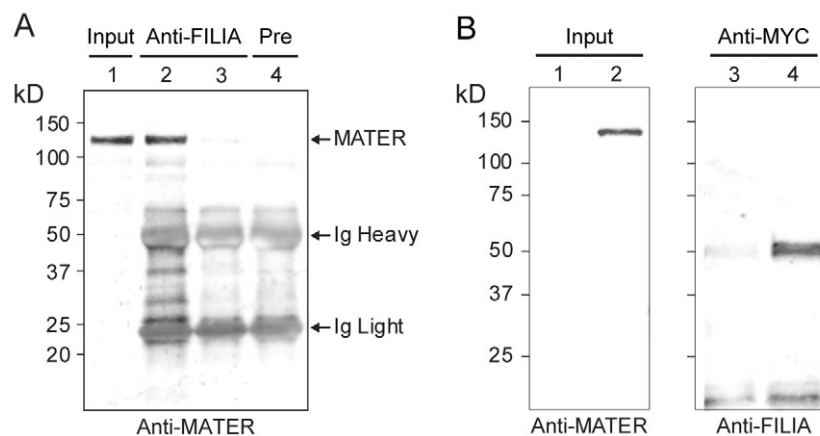


Fig. 7. FILIA interacts with MATER. (A) Immunoprecipitation of MATER with antibodies to FILIA. Ovulated eggs (~120) from wild-type (lanes 1,2,4) or *Mater^{tm1tm}* (lane 3) mice were lysed (0.5% Triton X100 in PBS) and either analyzed directly (lane 1) or immunoprecipitated with peptide-purified sheep antibodies to FILIA (lanes 2,3) or pre-immune sera (lane 4). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, incubated with peptide-purified rabbit antibodies to MATER and visualized with chemiluminescence. (B) Expression vectors containing full-length MATER (MYC-tagged) and FILIA were co-transfected into 293T cells (lanes 2,4). Controls (lanes 1,3) were co-transfected with the MYC vector lacking MATER and the FILIA expression vector. Whole cell lysates were analyzed before (left panel) or after (right panel) immunoprecipitation with antibodies to MYC. Proteins were resolved on SDS-PAGE gels, transferred to nitrocellulose and probed with antibodies to MATER (left) or FILIA (right panel). Molecular masses (kDa) are indicated at the left for both A and B.

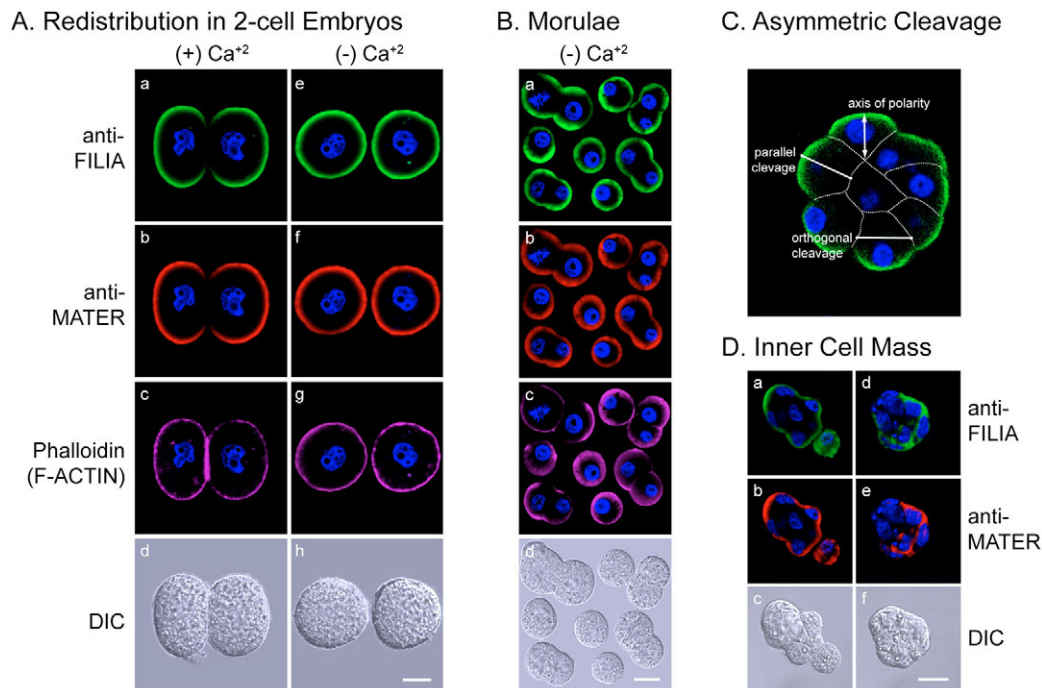


Fig. 8. Plasticity of FILIA and MATER localization during preimplantation development. (A) Zona-free two-cell mouse embryos were incubated with [(+) Ca^{2+} (a-d)] or without [(-) Ca^{2+} (e-h)] calcium to induce disaggregation. After fixation and permeabilization, embryos were stained with antibodies specific to FILIA (a,e), MATER (b,f) and phalloidin, which binds to F-actin (c,g), before imaging by confocal (a-c,e-g) or differential interference contrast (DIC) microscopy (d,h). (B) Same as A except individual and clumps of blastomeres were isolated from single zona-free morulae in the absence of calcium for localization of FILIA (a), MATER, (b), actin (c) or visualization by DIC microscopy (d). (C) Cell cleavage of blastomeres can be parallel or orthogonal to the axis of polarity. If parallel, the daughter cells are equivalent; if orthogonal, the 'outer' cells seemingly contain the FILIA-MATER complex and 'inner' cells do not. The image is from Fig. 6. (D) The ICM of early two (a-c; d-f) blastocysts was isolated by immunosurgery and imaged as in A with antibodies to FILIA (a,d) and MATER (b,e) or visualized by DIC (c,f). Normal blastocysts served as positive imaging controls (data not shown). Scale bars: 20 μm in A,B,D.

symmetrical segregation of cytoplasmic contents. Each protein initially is uniformly localized to the subcortex of growing oocytes, ovulated eggs and one-cell zygotes. As the embryo completes its first division, both proteins become excluded from the region of contact between the blastomeres and are asymmetrically restricted to the periphery. The co-localization and physical interaction of FILIA and MATER suggest common participation in a subcellular structure independent of F-actin (Fig. 8A) and of E-cadherin (data not shown) networks, from which they can physically separate. Cell divisions orthogonal to the apical-basal axis (Fig. 8C) of the polarized cell result in cell populations marked by the presence or absence of the FILIA-MATER complex (Fig. 6) even though the two component proteins persist in 'inner' cells of the morula and ICM of blastocysts (Fig. 8D). Thus, the complex, rather than individual FILIA and MATER proteins, serves as a cell-lineage marker during early embryogenesis.

These observations are consistent with a model in which the early embryo, although subject to regulative development, differentially accumulates a maternally expressed protein complex in topologically distinct blastomeres. The progeny of those containing the FILIA-MATER complex preferentially form the trophoblast; those without the complex preferentially become the ICM of the blastocyst. Other proteins also adopt a polarized location during preimplantation development, some as early as the two-cell stage, such as T-cell leukemia/lymphoma 1 (TCL1) and ubiquitin carboxy-terminal hydrolase L1 (UCHL1) (Narducci et al., 2002; Sekiguchi et al., 2006), and others not until the eight-cell stage of

embryogenesis, such as ezrin (VIL2 – Mouse Genome Informatics) and PAR3-aPKC. Ezrin, a member of the ezrin-radixin-moesin (ERM) family of proteins (Sato et al., 1992) was reported as an early marker of blastomere polarization and of trophoblast precursor cells (Louvét et al., 1996; Dard et al., 2001). It is widely expressed in the mouse, where it localizes to the apical cortex of epithelial cells (Berryman et al., 1993). Although not essential for development (Saotome et al., 2004), ezrin, derived from maternal and zygotic transcripts, remains symmetrically present at the periphery of blastomeres until the eight-cell stage, when it becomes confined to the apical cytocortex. The PAR3-aPKC complex is also asymmetrically located in the eight-cell embryo, and experimental disruption of either protein in individual blastomeres *in vitro* partially redirects their cell fate toward that of an 'inner' cell after the fourth embryonic cleavage (Plusa et al., 2005). Whether these proteins interact with the FILIA-MATER complex beginning at the eight-cell stage of development has not been ascertained.

The ability of the FILIA-MATER complex to redistribute during early cleavage stages of embryogenesis is particularly striking. At minimum, the apical cytocortical localization of the FILIA-MATER complex serves as an early marker of subsequent cell-fate commitment, although it may have functional significance in the establishment of cell lineages. One could envision a role for the FILIA-MATER complex in sequestering macromolecules that eventually trigger, either directly or indirectly, a commitment of the 'outer' cell progeny to become trophoblasts (Johnson and McConnell, 2004). The ability of some 'inner' cells to become

'outer' cells with trophectoderm descendants after incubation with morulae (Ziomek and Johnson, 1982) may reflect the restoration of the FILIA-MATER complex in the absence of cell-cell contact, as observed after immunosurgery (Fig. 8D). Alternatively, the complex may participate in maintaining the totipotency of cleavage-stage blastomeres. In theory, initial cell lineages (trophectoderm versus ICM) could be established at the two-cell stage, but by deferring the dichotomous divide until eight cells, the risk of catastrophe from the loss or damage of a single cell is substantially reduced. However, this requires totipotency during the first three cell divisions and compensatory mechanisms for the loss of a single blastomere that could involve the plasticity of FILIA-MATER complex localization. Following asymmetric cell division at the eight-cell stage, absence of the FILIA-MATER complex in the 'inner' cells may release them from totipotency and initiate lineage decisions in the ICM. Embryos derived from *Mater^{tm/tm}* females arrest at the two-cell stage, and their subsequent lethality suggests a checkpoint during the early totipotent cleavage stages. This formulation would be consistent with models in which cell fate is determined by cleavage pattern and positions of blastomeres in the embryo (Wilson et al., 1972) and with the more recent observation that depletion of PAR3 predisposes blastomeres to become 'inner' cells (Plusa et al., 2005).

In an earlier screen of mouse embryonic stem cells using digital differential display, *Filia* transcripts were identified as ES cell associated transcript 1 (*Ecat1*) expressed in embryonic stem cells, but not in 12 other tissues, which did not include the ovary (Mitsui et al., 2003). More recently, *Ecat1* transcripts have been observed in induced pluripotent stem (iPS) cells derived from skin (Okita et al., 2007). The presence of protein product, however, was not reported, and ectopic expression of ECAT1 in embryonic fibroblasts did not consistently elicit embryonic stem marker gene expression, although other ECAT proteins did (Takahashi and Yamanaka, 2006). FILIA is conserved among mammals with a rat homolog of 434 amino acids with ten repeats (67% amino acid identity) and a shorter human homolog (217 amino acids, 41% identity) with only four repeats. Studies are underway to genetically ablate the single copy *Filia* gene to investigate its function in early mouse embryogenesis.

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