Participation of EML6 in the regulation of oocyte meiotic progression in mice

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Abstract

The generation of a high-quality egg for reproduction requires faithful segregation of chromosome during oocyte meiosis. Here, we report that echinoderm microtubule-associated protein like 6 (EML6) is highly expressed in oocytes, and responsible for accurate segregation of homologous chromosomes in mice. Quantitative real-time RT-PCR and immunohistochemistry analyses revealed that EML6 was predominantly expressed by oocytes in the ovaries. Whole mount immunofluorescent staining showed that EML6 was colocalized with spindle microtubules in oocytes at various stages after meiotic resumption. This specialized localization was disrupted by nocodazole, the microtubule destabilizer, while enhanced by Taxol, a microtubule stabilizing reagent. In vivo knockdown of Eml6 expression by the specific siRNA resulted in chromosome misalignment and alteration in spindle dimension at both metaphase I and II stages, as well as the increased aneuploidy in the mature oocytes. Thus, these data suggest that EML family proteins participate in the control of oocyte meiotic division.

Keywords: echinoderm microtubule-associated protein like 6 (EML6), meiosis, spindle, chromosome alignment, oocyte aneuploidy, female fertility

Introduction

To produce an egg competent in fertilization and embryo development, mammalian oocytes must complete meiosis for the recombination and segregation of homologous chromosomes[1]. The precise timing and accurate chromosomal segregation of meiosis are therefore key determinants of oocyte quality. However, for most mammalian species, especially the human, meiosis in oocytes is error-prone, because it frequently causes aneuploidy in the resulting eggs and embryos which is a leading cause for female infertility, miscarriage, and birth defects. As the incidence of aneuploidy rises significantly with the maternal age, there is growing interest in the fundamental mechanisms governing oocyte meiosis and the underlying causes of oocyte aneuploidy[2].

Faithful segregation of chromosomes during oocyte meiotic maturation is heavily dependent upon the formation of a well-organized bipolar spindle apparatus, and the correct interactions between chromosomes and microtubules[3]. Microtubules are made up of hollow cylindrical polymers of α/β-tubulin subunits. These highly dynamic microtubules can...
switch stochastically between growing and shrinking phases both in vivo and in vitro\(^4\). The dynamic function of spindles requires the interaction of microtubules with a large number of proteins. These microtubule-associated proteins (MAPs) regulate the stability status of microtubules. For example, some MAPs, such as MAP2\(^5\), Tau\(^6\), and CKAP2\(^7\), promote microtubule growth via stabilizing them; while others, such as stathmin/Op18\(^8\), katanin\(^9\), and XKCM1\(^10\), are microtubule-destabilizing proteins that promote microtubule disassembly. To date, over 200 MAPs are identified to be essential for spindle assembly in somatic cells\(^11\). However, the composition and function of the meiotic maturation-requiring MAPs in oocytes remain undefined.

Recently, we investigated the potential involvement of mammalian homologs of echinoderm microtubule-associated protein (EMAP), EMLs (EMAP-like), in the control of mouse oocyte meiotic progression. Attention was paid specifically to this group of proteins owing to their uniqueness among the known MAPs. The founding member of the EML family is EMAP, which was first identified in 1993 as the most abundant microtubule-binding protein in sea urchin eggs and embryos\(^[12–13]\). The name “EMAP” was then assigned to this protein because of its robust expression in sea urchin, starfish, and sand dollar eggs\(^[12]\). The six members of the mammalian EML are highly similar in sequence, and all contain a unique hydrophobic EML protein (HELP) domain required for microtubule binding and several WD40-repeat-containing domains (Fig. 1). Due to lower sequence homology with other known MAPs, such as those in the brain, the mammalian EMLs are considered to be a unique class of MAP. Biochemical studies have indicated that some members, such as EML4, promote microtubule stabilization\(^[14–16]\), while others (e.g. EML2) destabilize microtubules\(^[17]\). Most EMLs were found to be expressed in the nervous system except EML3, which was also found to be expressed in the liver and the kidney\(^[14–15,18–21]\). Despite this yet scant information on the expression and function of EML family members, virtually nothing was known on the expression patterns and the potential roles of EMLs in the oocytes.

Here we reported that EML6 is the only member of the EML family that is preferentially expressed by oocytes in mouse ovaries. EML6 is co-localized with meiotic spindles, and this specialized localization is crucial for the maintenance of spindle integrity and the fidelity of homologous chromosome segregation. To the best of our knowledge, this is the first study to demonstrate the expression and function of the EML family member in meiotic maturation of mammalian oocyte.

Materials and methods

Chemicals and reagents

Unless otherwise specified, all chemicals and reagents were purchased from Sigma-Aldrich (USA).

Animals

Specific pathogen-free (SPF) ICR female mice (9–11 g in weight) were used for all the experiments. These mice were purchased either from Nanjing Medical University Animal Core Facility or Beijing Vital River Laboratory Animal Technology Co., Ltd. All experimental and animal procedures were approved by the Ethical Committee of Laboratory Animals and the Animal Care and Use Committee of

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Fig. 1  Schematic illustration of the domain composition of mouse EML family proteins. The mouse EML family has six members, EML1-6, which contain two domains: the HELP domain (dark red) and the WD40-repeat-containing domain (dark blue). The position of each domain within the amino acid (aa) sequence of each protein is indicated by the numbers above the corresponding sequences.
Nanjing Medical University, and performed in accordance with the institutional guidelines.

Oocyte, cumulus and mural granulosa cell collection, and oocyte culture

Female mice were primed with 5 U of pregnant mare serum gonadotropin (PMSG) (Ningbo Second Hormone Factory, China) to stimulate the antral follicle development in the ovaries. Ovaries were picked up 46–48 hours after the initial priming, and placed into a 3 cm dish containing MEM-alpha (Gibco, USA) medium. Large antral follicles were then punctured with a pair of syringes connected with 25 gauge needles to release the mural granulosa cells (MGCs) and cumulus-oocyte complexes (COCs). COCs were collected, and oocytes and cumulus cells were separated by repeatedly pipetting the COCs using a mouth controlled glass pipette with the inner diameter slightly smaller than the oocyte. Similar amount of MGCs, cumulus cells (CCs), and germinal vesicle (GV) stage fully-grown oocytes (FGOs) were then collected. These procedures were carried out in MEM-alpha supplemented with 75 μg/mL penicillin G, 50 μg/mL streptomycin sulfate, 25 μg/mL pyruvate, 3 mg/mL bovine serum albumin (BSA) and 5 μmol/L milrinone (Calbiochem, Germany), a specific inhibitor of PDE3A to maintain oocyte meiotic arrest.

For oocyte culture, GV-stage oocytes were incubated in MEM (Gibco) supplemented with 75 μg/mL penicillin G, 50 μg/mL streptomycin sulfate, 25 μg/mL pyruvate, 38 μg/mL EDTA and 3 mg/mL BSA. Culture was conducted at 37 °C in an incubator with 5% O₂, 5% CO₂ and 95% N₂. Oocytes at GV, Pro-metaphase I (Pro-MI), metaphase I (M I), anaphase I (A I)/telophase I (T I), and M II stages were collected at 0, 4, 6–7, 8–10, and 12–14 hours after the culture was set up.

qRT-PCR analysis

Total RNA was extracted from granulosa cells and oocytes using RNasy micro kit (Qiagen, Germany), and in vitro transcription and real-time PCR analyses are then carried out using QuantiTect® Reverse Transcription Kit (Qiagen) and QuantiTeck® SYBR Green PCR Kits (Qiagen), respectively, on the ABI 7500 Real-time PCR System (Applied Biosystems, USA). The relative fold changes in mRNA levels were calculated via the method of 2^−ΔΔCt using Rpl19 as an internal control. The primer sequences for the tested genes are listed in Table 1.

Ovarian immunohistochemistry and immunofluorescence

Isolated mouse ovaries initially primed with PMSG for 46 hours were fixed for 4 hours in 4% paraformaldehyde, and then embedded in paraffin and sectioned at 5-μm thickness for immunohistochemistry and immunofluorescence. Immunostaining of EML6 was then carried out using rabbit anti-EML6 primary antibody (1:100), and the VECTASTAIN ABC-AP KIT (Rabbit IgG, catalog No. AK-5001, Vector, USA) and VECTOR Red Alkaline Phosphatase (AP) Substrate Kit (catalog no. AK-5100) purchased from Vector Laboratories. Alexa Fluor-488 donkey anti-rabbit IgG was used as the secondary antibody for immunofluorescence.

Whole mount immunofluorescent staining of oocytes

Oocytes were fixed in 4% paraformaldehyde for 30 minutes at room temperature, and then washed and blocked in PBS containing 10% fetal bovine serum (FBS) and 1% Triton X-100 for 1 hour at 37 °C or at 4 °C overnight. After blocking, oocytes were incubated overnight at 4 °C with EML6 antibody (1:100) and FITC-α-tubulin antibody (1:500, Proteintech, Wuhan, China). After three washes with PBS-1% FBS, oocytes were incubated for 2 hours in Alexa Fluor 594-conjugated secondary antibody solution (1:200), and finally counterstained with DAPI (1:100) for 10 minutes, and mounted on glass slides for imaging using a laser scanning confocal microscope (LSM 700, Carl Zeiss, Germany).

Nocodazole and Taxol treatment

After 8 hours in culture, oocytes at M I stage were transferred to pre-warmed culture medium containing 20 μg/mL nocodazole or 10 μmol/L Taxol, and incubated for 10 or 45 minutes, respectively.

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**Table 1 Primers used for real-time RT-PCR analysis**

| Primers  | Sequence (5′→ 3′) |
|----------|--------------------|
| EML1-F   | GCACATCTAAGGATGGAAGCAA |
| EML1-R   | CGGTCAAAAAAGCCTATTCAA |
| EML2-F   | CCCGCACCTGTAAGCAA |
| EML2-R   | CAAATCCCACAACACTCAA |
| EML3-F   | ATTTGCATCCATGACAAACATGA |
| EML3-R   | TGATAAAACTGGATGTCGCCATA |
| EML4-F   | GACGCCAGTGTAACAAAAAC |
| EML4-R   | GCCCATCTGCTTTCTCTT |
| EML5-F   | CATGTTGCGCTACCGCTGCT |
| EML5-R   | AGGGAGGCGGACACATCCAC |
| EML6-F   | AGAAAAAGGCCACACC GGATCGCC |
| EML6-R   | ATGATGTCGCCACCATCGTT |
oocytes were incubated with the same concentration of dimethyl sulfoxide (DMSO) under the same culture conditions. After treatment, the oocytes were washed thoroughly and fixed for immunofluorescence.

**Chromosome spread**

Zona pellucida of MII-stage oocytes was removed by exposure to acid Tyrode's solution (pH 2.5) for 30 seconds at 37 °C. The oocytes were put on a glass slide in a small drop of fixative containing 1% PFA and 0.15% Triton X-100, and then air dried. The fixed oocytes were incubated with the Human anti-Centromere (1:500, Antibodies Incorporated, USA) at 4 °C overnight after being blocked with 1% BSA for an hour, followed by incubation with Alexa Fluor594-conjugated donkey anti-Human IgG (1:750, Invitrogen, Shanghai, China). The chromosomes were counterstained with Hoechst 33342 for 15 minutes. The specimens were examined under a LSM 700 laser scanning confocal microscope (Carl Zeiss).

**Statistical analysis**

All experiments were repeated at least three times independently, with the data presented as mean ± SEM. Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, USA). Differences between the groups were analyzed using Student's t-test. *P*<0.05 was considered to be significantly different.

**Results**

**Robust expression of EML6 in mouse oocytes**

As an initial step to explore the involvement of the mammalian homologue of EMAP in oocyte and ovarian biology, we examined the expression of all six members of the EML family in different compartments within the mouse ovarian follicles. Quantitative real-time RT-PCR analysis revealed that EMLs displayed various patterns of differential expression within the follicles (*Fig. 2A*). In oocytes, EML4, EML5, and EML6 were expressed at much higher levels than EML1, EML2, and EML3, with EML4 being the most abundantly expressed in oocytes. In follicular somatic cells, i.e., cumulus and mural granulosa cells (CCs and MGCs), all the EMLs were abundantly expressed except that EML6, and EML3 and EML5 were expressed relatively lower than the other three. Interestingly, only EML6 was found to be predominantly expressed by oocytes. The levels of EML6 mRNA in oocytes were 27 and 14 folds higher than those expressed in CCs and MGCs, respectively. Consistent with the preferential expression of the mRNA, immunohistochemistry analysis demonstrated that EML6 protein was also robustly expressed in the oocytes, but barely detected in ovarian somatic cells, including the CCs and MGCs (*Fig. 2B*).

**Specialized localization of EML6 during oocyte meiotic maturation**

The predominant expression of EML6 in oocytes implies its important role during oocyte maturation. We therefore investigated the intracellular localization of EML6 during oocyte meiotic progression. As indicated in *Fig. 3A*, whole mount immunofluorescence revealed that EML6 was ubiquitously distributed in the cytoplasm of GV-stage FGOs. After GV breakdown (GVBD), it was enriched at the spindle region, and co-localized with spindle microtubules at all the meiotic stages that were examined. To ascertain whether the specialized localization of EML6 was dependent on the integrity of the spindle apparatus, M1 stage oocytes were treated briefly with spindle de-stabilizer nocodazole and stabilizer Taxol, respectively, and the localization of EML6 was then examined (*Fig. 3B*). Unlike DMSO treatment which did not affect the size and shape of the M1 spindles or EML6 localization, nocodazole treatment disrupted the normal structure of the meiotic spindles, which is coincident with the dissociation of EML6 from the collapsed spindle apparatus. However, Taxol stabilized the M1 spindles, and enhanced the co-localization of EML6 with the spindle microtubules.

**Impairment of spindle morphology and chromosome alignment in EML6 deficient oocytes**

The specific localization of EML6 on spindles suggests that it may control oocyte meiotic maturation. We tested this possibility by knocking down (KD) the expression of EML6 in GV-stage FGOs and assessing the consequence on oocyte meiotic progression (*Fig. 4A*). As shown in *Fig. 4B*, the levels of EML6 mRNA in oocytes were downregulated by 92.5% after receiving the microinjection of EML6-specific small interfering RNAs (siRNAs). GVBD occurred normally, and completed within 1.5–2.0 hours after being released from the milrinone-containing medium in EML6-KD oocytes (*Fig. 4C*), indicating that EML6 KD did not affect oocyte meiotic resumption. However, the kinetics of the first meiosis, as assessed by emission of the first polar body (PB1), was significantly delayed in EML6-KD oocytes (*Fig. 4D*). When the morphology of spindle apparatus and chromosome dynamics were assessed by whole mount

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IF staining, evident impairment of the alignment of chromosomes and the dimension of spindles was observed in the EML6-KD oocytes both at the M I and M II stages (Fig. 5). Chromosomes were not properly aligned at the spindle equators, and instead were scattering around in EML6-KD oocytes, which resulted in a wider metaphase plate at both the M I - (Fig. 5B) and M II -stages (Fig. 5G). The dimension of meiotic spindles was also distorted in EML6-KD oocytes, with the spindle length and area increased in
MI-stage (Fig. 5C and E), while in MII only the width was enlarged (Fig. 5I). Other parameters of the spindles were not changed at both stages (Fig. 5D, H, and J).

**Activation of spindle assembly checkpoint and increases of aneuploidy in EML6-KD oocytes**

The precedent defects in spindle morphology and chromosome alignment observed in EML6-KD
oocytes implied that spindle assembly checkpoint (SAC) and the fidelity of homolog separation might also be affected in these oocytes. We therefore tested this possibility by assessing the activation status of SAC in EML6-KD oocytes at M I-stage and the euploidy status of the EML6-KD oocytes that have completed the first meiotic division, respectively. IF staining of BubR1, the SAC component, in oocytes that had been cultured for 7 hours demonstrated that the percentage of chromosomes stained positive for BubR1 was significantly higher in EML6-KD oocytes (Fig. 6A and B), indicating that SAC was constantly active in EML6-KD oocytes when meiosis normally progressed to M I in the control oocytes. Chromosomes spread analysis of the oocytes that have extruded the PB1 revealed that most of the control oocytes had 20 chromosomes, and there were only 5% aneuploid oocytes. In contrast to the control, the EML6-KD oocytes had significant elevated rate of aneuploidy, which increased to nearly 30% (Fig. 6C and D).

Discussion

Maintenance of the normal structural and functional dynamics of the meiotic spindle is indispensable for meiotic progression and chromosome segregation in mammalian oocytes. Here, we revealed for the first time that the EML family of proteins, a unique class of MAPs, are differentially expressed in the ovarian follicles of mice, with EML1-3 highly expressed by granulosa cells whereas EML4-6 robustly expressed by oocytes. EML6 is the only member that is both abundantly and preferentially expressed by oocytes. EML 4 and 5 were abundantly expressed by both granulosa cells and oocytes, with relatively higher levels in oocytes. The compartmentalization of the EML family members in the ovary implies that they may play unique roles in the control of oocyte and granulosa cell development. These data are therefore very informative for exploring the function
of EMLs in mammalian ovaries.

Given the robustness and preferential pattern of expression of EML6 in oocytes, we further explored its localization and potential function in oocytes. We found that EML6 was co-localized with spindle microtubules at various stages of meiotic progression after the oocyte resumes meiosis. This is consistent with the spindle localization of most of EML family members during mitosis in somatic cells[16,22]. This meiotic spindle-specific localization was lost when the nucleation of microtubules were disrupted when the oocyte is treated with nocodazole, the microtubule destabilizer; whereas the localization of EML6 on spindles was enhanced by treatment with Taxol, the

**Fig. 5** Effects of knockdown of EML6 on spindle configuration and chromosome alignment in oocytes. A: Representative images showing the morphology of spindles (green) and the alignment of chromosomes (red) in M I oocytes of the control and EML6-KD groups. B–E: Quantitative assessment of chromosome alignment (B) and spindle morphology (C–E) in M I oocytes of the control and EML6-KD groups. F: Representative images showing the morphology of spindles (green) and the alignment of chromosomes (red) in M II-stage oocytes of the control and EML6-KD groups. G–J: Quantitative assessment of chromosome alignment (G) and spindle morphology (H–J) in M II oocytes of the control and EML6-KD groups. Scale bar=10 μm. *P<0.05, **P<0.01, ***P<0.005, compared with the control. “ns” indicates no significant differences observed between the control and EML6-KD groups.
microtubule stabilizing reagent. These data further buttress the association of EML6 with the microtubules during oocyte meiotic progression, and strongly suggest the participation of EML6 in the control of oocyte meiotic maturation. Indeed, we found that the knockdown of EML6 delayed the progression of meiosis toward metaphase II, as indicated by the slower kinetics of extrusion of PB1, although GVB was not affected. This suggests that EML6 is essential for the orderly progression and completion of the first meiosis, but dispensable for the re-initiation of meiosis.

Spindle size and chromosome alignment at spindle equators were also found to be severely impaired in EML6-KD oocytes at both the M I- and M II-stages. This suggests that EML6 is crucial for the assembly and/or maintenance of normal spindles, and the congression of chromosomes during the oocyte meiotic maturation. In accord with the phenotype of chromosome misalignment at M I stages, the rate of BubR1-positive chromosomes within each oocyte was significantly upregulated in EML6-KD oocytes, thus indicating the constant activation of SAC. Therefore, the delay in PB1 extrusion after EML6 knock-down could be explained nicely by chromosome misalignment and SAC activation at M I-stage. Defective segregation of the homologous chromosomes at the end of the first meioses is frequently reported to lead to chromosome misalignment in the resulting M II-stage oocytes[23–24]. Indeed, we found here that, associated with the defective chromosome alignment and spindle configuration in EML6-siRNA treated oocytes, the rate of aneuploidy was also increased significantly. This observation suggests that EML6 is also essential for the fidelity of homolog segregation during the final stage of oocyte meiotic maturation. The similar role in chromosome alignment and spindle morphology has also been discovered for EML1, EML3, and EML4 during mitosis in somatic cells[16,22,25], indicating that the members of mammalian EML family play a conserved role in the control of both mitosis and meiosis.

Taken together, although the founding member of EMLs was originally identified from sea urchin eggs and embryos, the participation of mammalian EMLs in the control of oocyte maturation has not been...
explored previously. It is anticipated that the results presented here will inspire more interest in understanding the roles and related mechanisms of EMLs in the control of oocyte development and maturation.

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