

Spastin Interacts with CRMP5 to Promote Spindle Organization of Mouse Oocytes by Severing Microtubules.

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Research article

Keywords: Spastin Interacts with CRMP5 to Promote Spindle Organization, Mouse Oocytes by Severing Microtubules

Posted Date: February 28th, 2020

DOI: <https://doi.org/10.21203/rs.2.24806/v1>

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Abstract

Background Microtubule-severing protein (MTSP) is highly critical for the survival of both mitotic and post-mitotic cells. However, the study of MTSP in the meiosis of mammalian oocyte has not been reported.

Results We found that spastin, a member of the MTSP family, was highly expressed in oocyte and aggregated in spindle microtubules. After knocking down spastin by specific siRNA, the spindle microtubule density of meiotic oocyte decreased significantly. When the oocyte was cultured in vitro, the oocyte lacking spastin showed obvious maturation obstacles. Combining with the microtubule severing activity of spastin, we speculate that spastin on spindle may increase the microtubule broken ends by severing microtubules, thus playing a nucleating role, promoting spindle assembly and ensuring normal meiosis. In addition, we found that there was co-localization and interaction between CRMP5 and spastin in oocyte. The knockdown of CRMP5 may also lead to spindle abnormalities and developmental disorders in oocyte. Overexpression of spastin may save the abnormal phenotype caused by deletion of CRMP5.

Conclusions To sum up, our data support a model in which the interaction between spastin and CRMP5 promotes the assembly of spindle microtubules in oocyte by controlling microtubule dynamics, thus ensuring normal meiosis.

Background

Microtubule-severing proteins (MTSPs) generate internal breaks in microtubules (MTs). They are conserved in eukaryotes from ciliates to mammals, and their function is important in diverse cellular processes ranging from cilia biogenesis to cell division, phototropism, and neurogenesis. MTSPs are categorized into the “meiotic” subfamily of ATPases associated with diverse cellular activity (AAA) superfamily (1, 2). All MTSPs have a AAA domain that can bind and hydrolyze ATP to sever a MT along its length (1-4). Thus, MT severing is a more efficient way to reorganize MTs when compared with end-limited MT depolymerization by kinesins (5-10).

Abnormal chromosome segregation during mitosis can lead to aneuploidy, which is one of the main causes of tumorigenesis (11,12). Many studies have shown that MTSPs play an important role in mitosis through their MT severing activity. Katanin 60, the first discovered MTSP, target the centrosome and promote the aggregation of γ -tubulin on the spindle, thus promoting the formation of microtubule organizing center (MTOC) (13,14). The second identified MTSP member, spastin, was found to be the most common form variant of autosomal dominant hereditary spastic paraplegia (15). Spastin was later shown to be localized in the centrosome during mitosis and play an important role in centrosome-based mitosis (16,17). Fidgetin (16,18), fidgetin-like 1 (19), katanin-like 1 (20) and katanin-like 2 (21) are also considered essential for mitosis. They show different localization patterns in cells, and the absence of these proteins affects the assembly of spindle microtubules in varying degrees.

Compared with mitosis, the process of meiosis is unique. Meiosis consists of a single DNA replication and two rounds of cell division, forming haploid gametes. There is no typical centrosome in oocyte or spermatocyte, but some centrosome components have been found at the two poles of spindle. There may be different regulation mechanisms in meiosis and mitosis. Normal meiosis is essential for genomic stability, and the main cause of human genetic diseases and early abortion is aneuploidy caused by dysfunction of oocyte division (22-24). The role of MTSPs in female meiosis in mammals is rarely reported, and mammalian meiosis is usually used as a model of human meiosis.

As a member of microtubule severing protein family, spastin also plays an important role in cell division and neurite growth, which are closely related to MTs (25,26). Spastin gene mutations were found to be the most common cause of autosomal dominant hereditary spastic paraplegia (HSP) (15). In the axons of nerve cells, spastin tends to sever long microtubules into short segments, releasing additional aggregation terminals. This severing activity makes the microtubules more vigorous, promotes the formation of neurite branches, and promotes the growth and regeneration of neurites. Spastin overexpression significantly increased the formation of neurite branches, while spastin depletion resulted in a significant decrease in the formation of axonal collateral branches but did not significantly reduce the length of axons (27,28). The results showed that the expression of spastin in neurons was regulated by transcription factor ELK1. ELK1 could bind to the key promoter regions of katanin-60 and spastin, and methylation in this region could lead to the decrease of ELK1 binding and the increase of the transcription of corresponding genes (29). In addition, spastin has been shown to interact with collapsin response mediator protein 5 (CRMP5), which provides structural support and promotes microtubule aggregation as a transportation route. Spastin interacts with CRMP5 to promote outward growth of neurites by controlling microtubule dynamics (30). Spastin's microtubule binding activity-related site has been located in the 59th amino acid residue (N-terminal region of AAA domain), which is known as the microtubule binding domain (MTBD) (31). The combination of CRMP5 and spastin in this region can increase the microtubule severing activity of spastin. During somatic mitosis, spastin is concentrated in the centrosome of cells. This localization is regulated by centrosome protein NA14 (16). Spastin can provide more free ends on the centrosome through microtubule-severing activities, which promotes the depolymerization of microtubules at spindle poles, thus separating chromosomes from each other (16,17).

However, during the meiosis of mammalian germ cells, spastin has never been studied before. Our focus is whether the distribution and function of spastin in mouse oocyte are consistent with that of somatic cell. Does the mechanism of spastin protein expression and spastin function regulation coincide with previous studies?

Methods

General chemicals, reagents cells and animals. Chemicals and reagents were obtained

from Sigma-Aldrich; Merck KGaA unless otherwise stated. The NIH3T3 cell line was purchased from the American Type Culture Collection. A total of 265 3-week-old female specific pathogen free ICR mice (weighing 18-20 g) used in this study were obtained from Vital River Experimental Animal Technical Co., Ltd. Animals were housed at a temperature of 20-26°C and a humidity of 40-70% with a 12 h light/dark cycle. The mice were fed in feeding boxes, and the frequency of food replacement was 2 times a week, and the frequency of water bottle replacement was 3 times a week. All animal experiments were approved by the Animal Care and Use Committee of Nanjing Medical University (Nanjing, China) and performed in accordance with institutional guidelines.

Antibodies. Mouse monoclonal anti- β -actin (cat. no. A5316-100) antibody was obtained from Sigma-Aldrich; Merck KGaA. Mouse monoclonal anti-SPASTIN (A-4) (cat. no. sc-398264) and mouse monoclonal anti- β -tubulin (cat. no. sc-5274) antibodies were purchased from Santa Cruz Biotechnology, Inc. Human anti-centromere CREST antibody (cat. no. 15-234) was purchased from Antibodies Incorporated. Rabbit polyclonal anti-CRMP5 (cat. no. ab36203) was purchased from Abcam. Cy2-conjugated donkey anti-mouse IgG (code no. 715-225-150), rhodamine (TRITC)-conjugated donkey anti-goat IgG (code no. 705-025-147), and Alexa Fluor 647-conjugated donkey anti-human IgG (code no. 709-605-149) were purchased from Jackson ImmunoResearch Laboratories, Inc. Horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (cat. no. 31402) and HRP-conjugated goat anti-mouse IgG (cat. no. 31430) were purchased from Invitrogen; Thermo Fisher Scientific, Inc.

Oocyte collection and culture. Immature oocytes arrested in prophase I [germinal vesicle (GV) oocytes] were obtained from the ovaries of 3-week-old female ICR mice. The mice were first euthanized with CO₂ and then sacrificed by cervical dislocation, and the ovaries were isolated and placed in operation medium (HEPES) with 2.5 nM milrinone and 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.). Oocytes were released from the ovary by puncturing the follicles with a hypodermic needle. Cumulus cells were washed off the cumulus-oocyte complexes, and 50 isolated denuded oocytes were placed in 100- μ l droplets of culture medium under mineral oil in plastic dishes (BD Biosciences). The culture medium was MEM+ (MEM with 0.01 mM EDTA, 0.23 mM Na-pyruvate, 0.2 mM pen/strep, 3 mg/ml BSA and 20% FBS). The oocytes were cultured at 37.0°C, 5% O₂, and 5% CO₂ in a humidified atmosphere. Prior to *in vitro* maturation (IVM), all culture medium included 2.5 nM milrinone to prevent the resumption of meiosis.

siRNA production and microinjection. Sequences of all DNA templates used for siRNA production are listed in Table I. The sequence of the control templates was a mock sequence that did not specifically bind to any mRNA from the mouse genome. DNA templates against four different DNA coding (sequence coding for the amino acids in a protein, CDS) regions of Spastin siRNA were designed online through BLOCK-iT™ RNAi Designer (<http://rnaidesigner.invitrogen.com/rnaiexpress/>) with some modifications.

The sequence specificity was verified through a BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) homology search.

siRNAs were produced using the T7 RiboMAX™ Express RNAi System (Promega Corporation), according to the manufacturer's instructions. Briefly, for each double-stranded siRNA against one of the four Spastin CDS regions, two pairs of synthesized complementary single-stranded DNA oligonucleotides were first annealed to form two double-stranded DNA templates. Subsequently, two complementary single-stranded siRNAs were separately synthesized in accordance with these two templates and then annealed to form a final double-stranded siRNA. Next, the siRNA was purified by conventional phenol/chloroform/isopropanol precipitation and then aliquoted and stored at -80°C after a quality check on an agarose gel. A ready-to-use siRNA mixture was prepared by mixing the siRNAs against four target regions together at an equal molar ratio to a final concentration of 5 µM.

Microinjection of siRNA into the cytoplasm of fully-grown immature oocytes was used to knock down Spastin. After injections, oocytes were arrested at GV stage with 2.5 µM milrinone for 20 hours, and then were cultured in milrinone-free M2 medium for maturation.

Immunofluorescence. The oocytes were briefly washed in PBS with 0.05% polyvinylpyrrolidone (PVP), permeated in 0.5% Triton X-100/PHEM (60 mM PIPES, 25 mM HEPES pH 6.9, 10 mM EGTA, 8 mM MgSO₄) for 5 min and washed three times rapidly in PBS/PVP. Next, the oocytes were fixed in 3.7% paraformaldehyde (PFA)/PHEM for 20 min at room temperature, washed three times (10 min each) in PBS/PVP and blocked with blocking buffer (1% BSA/PHEM with 100 mM glycine) at room temperature for 1 h. Then, the oocytes were in sequence incubated at 4°C overnight with a primary antibody diluted in blocking buffer, washed three times (10 min each) in PBS with 0.05% Tween-20 (PBST), incubated at room temperature for 45 min with a secondary antibody diluted in blocking buffer (1:750 in all cases), and washed three times (10 min each) in PBST. Finally, the DNA was stained with 10 µg/ml Hoechst 33258 (Sigma-Aldrich; Merck KGaA) at room temperature for 10 min, and the oocytes were mounted onto a slide with mounting medium (0.5% propyl gallate, 0.1 M Tris-HCl, pH 7.4, 88% glycerol) and covered with a cover glass (thickness, 0.13-0.17 µm). To maintain the dimension of the oocytes, two strips of double-stick tape (thickness, 90 µm) were placed between the slide and cover glass. The primary antibodies were diluted as follows: anti-Spastin, 1:200; anti-tubulin, 1:500; anti-human kinetochore, 1:500. The oocytes were examined with an Andor Revolution spinning disk confocal workstation (Oxford instruments).

Western blotting. A total of 100 oocytes were lysed in Laemmli sample buffer (Bio-Rad Laboratories, Inc.) containing a protease inhibitor and boiled for 5 min before being subjected to 10% SDS-PAGE. The separated proteins were transferred to a PVDF membrane and then blocked in TBST (TBS containing 0.05% Tween-20) with 5% nonfat milk at room temperature for 1 h. Then, the PVDF membrane was separated and incubated overnight at 4°C with primary antibodies as follows: mouse monoclonal anti-β-actin (cat. no. A5316-100) was diluted with a blocking buffer (TBS containing 0.05% Tween-20) at a ratio of 1:1,000; Mouse monoclonal anti-SPASTIN (A-4) (cat. no. sc-398264) was diluted with a blocking buffer

at a ratio of 1:500 was diluted with a blocking buffer at a ratio of 1:2,000. After being washed in TBST, the membranes were incubated with HRP-conjugated rabbit anti-goat IgG or HRP-conjugated goat anti-mouse IgG (diluted with a blocking buffer to 1:1,000) for 1 h at room temperature and then processed using an ECL Plus Western Blotting Detection System (Vazyme). In the experiment, we used ImageJ 1.8.0 as data analysis software.

Data analysis and statistics. All experiments were repeated at least three times. Measurements on confocal images was performed with ImageJ (National Institutes of Health). Data are presented as the average \pm sem. Statistical comparisons were performed with Student's t-test in Excel. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Spastin is distributed in the spindle microtubules of MI and MII oocytes. Firstly, we detected the distribution of spastin in oocyte by immunofluorescence. It was found that in MI and MII stage oocyte, spastin aggregated on the spindle and co-localized with microtubules (Fig.1A). When the depolymerized microtubules were treated with nocodazole, the location of spastin disappeared (Fig.1B), which indicated that the location of spastin depended on microtubules, and it was not difficult to infer that its role was closely related to the spindle microtubules.

The absence of spastin results in maturation disorder and abnormal fertilization of oocytes. In order to explore the function of spastin in oocyte, we designed specific siRNAs and injected them into the developmental-inhibited GV oocytes to knockdown spastin. Western blot showed that spastin could be deleted by about 80% (Fig.2A). In vitro maturation (IVM) experiments showed that the oocytes lacking spastin showed obvious developmental disorder compared with the control group. When IVM for 2 hours, the proportion of germinal vesicle rupture (GVBD) in knock-down group was basically the same as that in control group (Fig.2B), but when IVM for 16 hours, the elimination rate of the first polar body in spastin-depleted group was significantly lower than that in control group (Fig.2C). The oocytes with first polar were fertilized in vitro with the sperm of normal male mice and the early embryos were cultured in vitro. It was found that two-cell (Fig.2D) and blastocyst rate (Fig.2E) in spastin-depleted group showed a significant decline. This indicates that the absence of spastin leads to the obstruction of oocyte maturation, and even for the so-called matured oocytes with first polar body, their ability of fertilization and early embryo development of has also been significantly reduced without spastin.

The absence of spastin resulted in a significant decrease in the number of spindle microtubules in MI and MII oocytes. In order to find out how spastin affects oocyte development, we developed spastin-deletion

oocytes into MI and MII stages in vitro. We found that the fluorescence intensity of spindle microtubule staining in spastin-deficient MI (Fig.3A) and MII (Fig.3B) oocytes was significantly lower than that in the control group, indicating that microtubule density decreased significantly at this time. The results of immunofluorescence image showed that the ratio of spindle to cytoplasmic area of oocyte lacking spastin was significantly smaller than that of control group, while the ratio of chromosome to spindle area was significantly increased (Fig.3C), indicating that deletion of spastin also led to the reduction of spindle. In addition, it was found that both the MI (Fig.3A) and MII (Fig.3B) oocytes deleting spastin showed the phenotype of lagging chromosome (Fig.3 arrow). This suggests that the abnormal structure of the spindle caused by the lack of spastin will affect the function of the spindle. The disorder of chromosome arrangement during meiosis will eventually affect the number of chromosomes in daughter cells, which is fatal to cell fate. The decrease of the number of spindle microtubules will inevitably affect the structure and function of the spindle. It is easy to explain the meiosis and maturation obstacles of oocyte.

Exogenous spastin can rescue oocyte dysplasia caused by CRMP5 deletion. In neurons, CRMP5 can bind to spastin at specific sites to promote the microtubule-severing activities. We know through experiments that this effect still exists in meiotic oocyte. Firstly, we demonstrated by immunofluorescence that CRMP5 was distributed in spindle microtubules in oocyte and co-localized with spastin (Fig.4A). Co-IP experiments show that both spastin and CRMP5 can precipitate and interact with each other (Fig.4B). When we knocked down CRMP5 in oocyte with specific siRNA (knock down percentage is shown in Fig.4C) and then performed in vitro maturation test, the elimination rate of the first polar body in knockdown group was significantly lower than that in control group when IVM16 hours (Fig.4D). In vitro fertilization and early embryo culture experiments showed that the rate of two-cell embryos from CRMP5-deficient oocyte fertilized with normal sperm was much lower than that of the control group (Fig.4E). It can be seen that the deletion of CRMP5 in oocyte is similar to that of spastin knockdown. When we knock down CRMP5 with specific siRNA and then inject the spastin mRNA transcribed in vitro into oocyte, the rate of oocyte maturation (Fig.4D) and two-cell embryos (Fig.4E) obtained by in vitro fertilization increased. It can be seen that exogenous spastin can rescue oocyte dysplasia caused by CRMP5 deletion.

Exogenous spastin can rescue the decrease of spindle density of oocyte caused by CRMP5 deletion. In the above experiments, we know that the deletion of CRMP5 can lead to abnormal oocyte development and is related to spastin. Does CRMP5 also affect spindle assembly? Our next experiments have also proved this point. The oocytes knocked-down CRMP5 developed to MI and MII stages in vitro. The fluorescence intensity of spindle microtubule staining was significantly reduced by immunofluorescence (Fig.5A and Fig.5B left). When spastin mRNA was microinjected into the oocyte which scavenged CRMP5, the fluorescence intensity of the spindle microtubules was significantly restored (Fig.5A and Fig.5B left). Similar to the phenotype of knockdown spastin, the lack of CRMP5 in oocytes also showed a significant decrease in the ratio of spindle to cytoplasmic area (Fig.5A and Fig.5B right). When exogenous spastin mRNA was supplemented, the ratio increased to a certain extent, but the difference was still significant

compared with the control group (Fig.5A and Fig.5B right). It can be seen that additional spastin mRNA can rescue the decrease of spindle MT density and spindle volume of oocyte caused by CRMP5 deletion in whole or in part. The role of spastin in promoting spindle assembly and cell maturation during oocyte development may be influenced and regulated by CRMP5.

Discussion

The process of oocyte development and maturation is essentially the process of meiosis of oocyte. As is known to all, oocytes only undergo accurate meiosis to achieve homologous chromosome recombination and separation and transmit the complete genetic information to offspring accurately and steadily, so as to successfully complete fertilization and support embryo development, and eventually produce healthy offspring. If an abnormal meiosis process results in an obstacle to oocyte maturation or an aneuploid due to uneven distribution of genetic material, some hereditary diseases such as Down's syndrome may occur in offspring (32-34). The early embryo development disorder caused by various reasons will cause embryo development to stagnate and eventually abort. Studies have shown that abnormal oocyte development is the main cause of early embryonic sterility (35). The meiosis of oocyte started as early as the migration of primordial germ cells to the reproductive ridge in the embryonic stage and stagnated at the double-line stage of prophase until the fetus grew up after birth and recovered before ovulation in the first group of follicles in adolescence. During meiosis, with the dynamic changes of spindle and chromosome structure, oocyte morphology undergoes GV, GVBD, MI, MII stages, eventually forming mature oocytes and obtaining fertilization ability. The spindle composed of microtubules is a very important structure for meiosis. The key to the whole meiosis process is the correct separation of homologous chromosomes under the traction of spindle microtubules. This process requires precise regulation of many proteins.

As an important component of the cytoskeleton, microtubules (MTs) are essential for almost all cell activities. MTs are also the main components of spindle, neuron axon, centrosome and other important cellular structures. Mutations in the tubulin coding genes can lead to serious defects in MT tissue and cause some serious human diseases (36-38). The key characteristic of MT is its dynamics and all functions of MT depend on it. During the interval of somatic cell division, MTs are stretched and shortened dynamically by MT polymerase and depolymerase at both positive and negative ends in order to realize intracellular substance transport, signal transduction and cell migration (39-41). At the cell division stage, MTs can form spindles to separate chromosomes by pulling in opposite directions. The activity of MTs is regulated by microtubule associated proteins (MAPs). Deletion of these proteins can lead to t related diseases (42-44). As an important group of MAPs, microtubule severing protein (MTSP) plays an important role in regulating MT activity (45,46). MTSP mainly utilizes the energy released by ATP hydrolysis to remove the protective caps of both positive and negative poles of MTs, thus exposing the free end of MTs to MT polymerase or depolymerase and realizing the dynamic change of MT elongation or shortening (47,48). Another major function of MTSP is "nucleation", which can sever a long MT into several short segments, so that the exposure of the free end of each segment of MT can generate a new long MT through the role of polymerase, thereby promoting the MT density of some cell structures

(49). It is noteworthy that mutation or deletion of MTSP can lead to severe hereditary diseases, neurological diseases, developmental disorders and low fertility in mice (50-53). Although the MTSP family shares a conservative AAA domain, the other regions of each member are completely different. Therefore, each MTSP has both synergistic and separate roles in functioning (48).

In this study, it was found that in mouse oocytes, the microtubule severing protein spastin was located in the spindle microtubule, which was different from that in the centrosome in somatic cells. Due to the lack of centrosome structure in oocytes, the spindle assembly based on centrosome cannot be realized. Spastin is located in the centrosome of somatic cells and plays a key role in the process of chromosome separation in the anaphase of mitosis by severing microtubules to change the dynamics of microtubules. Can spindle microtubule based spastin play a role in oocyte meiosis? Our experiment confirmed that the oocytes without spastin showed obvious developmental disorder, which was manifested in the decrease of the first polar body excretion rate after 16 hours of culture in vitro. In addition, when the oocytes excreting the first polar body were fertilized with the sperm from the normal male mice and cultured in vitro, the oocytes without spastin showed a significant decrease in the two-cell rate and blastocyst rate. Immunofluorescence results showed that the spindle microtubule density and spindle area of oocytes without spastin decreased significantly. Abnormal structure of the spindle caused by the lack of spastin affected the function of the spindle. Our study showed that a high proportion of lagging chromosome appeared in spastin deficient oocytes. The disorder of chromosome arrangement during meiosis will eventually affect the number of chromosomes in daughter cells, which will greatly affect the ultimate fate of oocytes. This suggests that spastin can promote spindle assembly by regulating spindle microtubule density, so as to ensure normal meiosis and oocyte development. Due to the defects of spindle structure, the oocytes lack of spastin show the problems of cell maturation and fertilization.

In somatic cells, spastin gathers in the centrosome at the two poles of spindle, that is, the region of microtubule organization center (MTOC). By severing activity, long microtubules are severed into short segments to provide more active ends. Microtubule polymerases can bind at the active end and play an enzymatic role to make microtubules elongate, thus increasing the density of spindle microtubules, which is also the nucleation of MTSPs. In the meiosis of oocytes, the spindle lacks the centrosome structure, and MTOC of the spindle is relatively scattered. We speculate that the aggregation of spastin on microtubules can still play a role in nucleation, cutting at the right place, providing more active ends of microtubules, thus increasing the density of microtubules and promoting the assembly of spindles. With a sufficient number of microtubules, spindle can play a perfect function, achieve the separation of homologous chromosomes. The development and maturity of oocytes can go on the right track.

As mentioned before, in nerve cells, CRMP5 binds to spastin in the microtubule binding domain (MTBD), regulating the microtubule severing effect of spastin, promoting outward growth of neurites by controlling microtubule dynamics. Is the microtubule cutting activity of spastin regulated by CRMP5 in oocytes? Our results showed that CRMP5 was co located with spastin in the spindle microtubules of oocytes. And the co IP experiment proves the interaction between them. When CRMP5 was knocked down, the density of spindle microtubules decreased, the development of oocytes was impaired and fertilization was

abnormal, which was consistent with the expression of oocytes in the absence of spastin. Moreover, the introduction of exogenous spastin can partially save these phenotypes, which further shows that CRMP5 can regulate the microtubule cutting activity of spastin in oocytes, which is similar to its role in nerve cells.

Conclusions

To sum up, this study reveals for the first time that Spastin, a member of MTSP, is concentrated on the spindle MTs during meiosis of mouse oocyte. Spastin knockdown results in the decrease of spindle microtubule density, which led to oocyte maturation failure and abnormal fertilization. The microtubule severing effect of spastin was regulated by CRMP5. The specific process that spastin affects spindle assembly by severing microtubules and the detailed mechanism of CRMP5 regulating spastin action need further study.

Abbreviations

MTSP microtubule-severing protein

MT microtubule

ATPases associated with diverse cellular activity AAA

MTOC microtubule organizing center

HSP hereditary spastic paraplegia

CRMP5 collapsin response mediator protein 5

MTBD microtubule binding domain

MAP microtubule associated proteins

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Animal Care and Use Committee of Nanjing Medical University (Nanjing, China) and performed in accordance with institutional guidelines.

Consent for publication

Not applicable

Availability of data and material

All data generated or analysed during this study are included in this published article

Competing interests

The authors declare that they have no competing interests.

Funding

This present study was supported by the Grant for Zhejiang Provincial Science and Technology Department (LQ19H040006). Zhejiang Provincial Foundation Committee provided financial support for this research and the preparation of the article. The corresponding author and the first author of this article played a key role in the research design, data collection, analysis and interpretation, report writing and the decision to submit the paper for publication as the sponsor.

Authors' contributions

HFS and LLG conceived and designed the experiments. LLG, ZJ, JWL, SSJ, WYC and YS performed the experiments. LLG analyzed the data. JWL, SSJ, HFS and LLG contributed the reagents/materials/analysis tools. HFS and LLG wrote the paper. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Acknowledgements

We thank Dr. Zhang (Hangzhou Medical College) for providing us with a mitochondrial probe.

Patient consent for publication

Not applicable.

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Table 1

Target Site	DNA templates
spastin CDS 557-581 ^a	Oligo1: GGATCCTAATACGACTCACTATAC <u>CCAAGGACCGTTTACA</u> ACTTCTAGA ^b
	Oligo2:AATCTAGAA <u>GTTGTAAACGGTCCTTGGT</u> TATAGTGAGTCGTATTAGGATCC ^b
	Oligo3: GGATCCTAATACGACTCACTATATCTAGAA <u>GTTGTAAACGGTCCTTGG</u> ^b
	Oligo4:AAGGTGAAGAATATGTCTGCCAGTGAG TATAGTGAGTCGTATTAGGATCC ^b
Spastin CDS 954-978 ^a	Oligo1: GGATCCTAATACGACTCACTATAGGACAGCAATCTTGCTAACCTTATA ^b
	Oligo2:AATATAAGGTTAGCAAGATTGCTGTCC <u>TATAGTGAGTCGTATTAGGATCC</u> ^b
	Oligo3: GGATCCTAATACGACTCACTATATATAAGGTTAGCAAGATTGCTGTCC ^b
	Oligo4:AAGGACAGCAATCTTGCTAACCTTATA <u>TATAGTGAGTCGTATTAGGATCC</u> ^b
spastin CDS 1475-1499 ^a	Oligo1: GGATCCTAATACGACTCACTATAAAGCTGTTCTCAGGCGTTTCATTA ^b
	Oligo2:AATTAATGAAACGCCTGAGAACAGCTTTATAGTGAGTCGTATTAGGATCC ^b
	Oligo3: GGATCCTAATACGACTCACTATA <u>TTAATGAAACGCCTGAGAACAGCTT</u> - ^b
	Oligo4:AAAAGCTGTTCTCAGGCGTTTCATTAATATAGTGAGTCGTATTAGGATCC ^b
spastin CDS 1698-1722 ^a	Oligo1: GGATCCTAATACGACTCACTATAGGTGAAGAATATGTCTGCCAGTGAG ^b
	Oligo2:A <u>ACTCACTGGCAGACATATTCTTCACCT</u> TATAGTGAGTCGTATTAGGATCC ^b
	Oligo3: GGATCCTAATACGACTCACTATA <u>CTCACTGGCAGACATATTCTTCACC</u> ^b
	Oligo4:AAGGTGAAGAATATGTCTGCCAGTGAGTATAGTGAGTCGTATTAGGATCC ^b
Control ^c	Oligo1: GGATCCTAATACGACTCACTATAC <u>CCTACGCCACCAATTT</u> CGTT ^b
	Oligo2:AAAAACGAAATTGGTGGCGTAGGTATAGTGAGTCGTATTAGGATCC ^b
	Oligo3: GGATCCTAATACGACTCACTATA <u>AAACGAAATTGGTGGCGTAGG</u> ^b
	Oligo4:A <u>ACCTACGCCACCAATTT</u> CGTTTATAGTGAGTCGTATTAGGATCC ^b

Table 1. DNA oligos for siRNA production.

¹ The numbers are the starting and ending position of the target sites in Spastin CDS (NM_001162870.1 in NCBI). ² two pairs of DNA oligos are needed for each double-stand siRNA. Oligo 2 is complementary with oligo 1 except an "AA" overhang at 5'; Oligo 3 is complementary with oligo 4 except an "AA" overhang at 5'. In each oligo, gene-specific sequences are underlined, other sequences are for recognition and binding by T7 RNA polymerase. ³ Control siRNA does not target to any mRNA sequence in mouse.

Figures

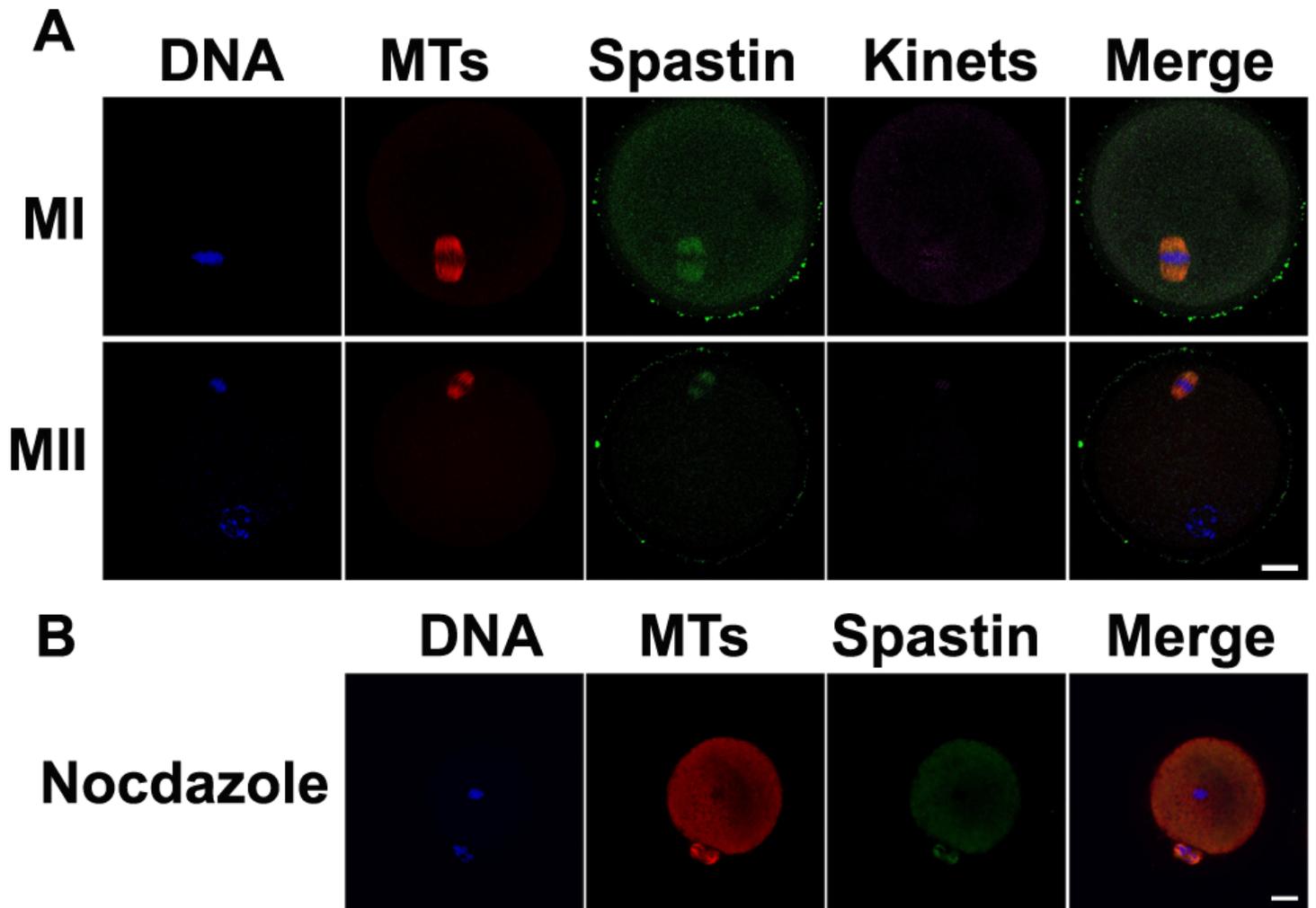


Figure 1

Spastin is located at the spindle microtubules of MI and MII oocytes. (A) Immunofluorescence revealed that spastin was located at the spindle microtubule of MI and MII mouse oocytes. (B) When nocodazole treated oocytes to depolymerize microtubules, immunofluorescence showed that the location of spastin disappeared with the disappearance of microtubules. Tubulin is displayed in red, Spastin in green, kinetochores in magenta, and DNA in blue. Scale bar, 20 μ m.

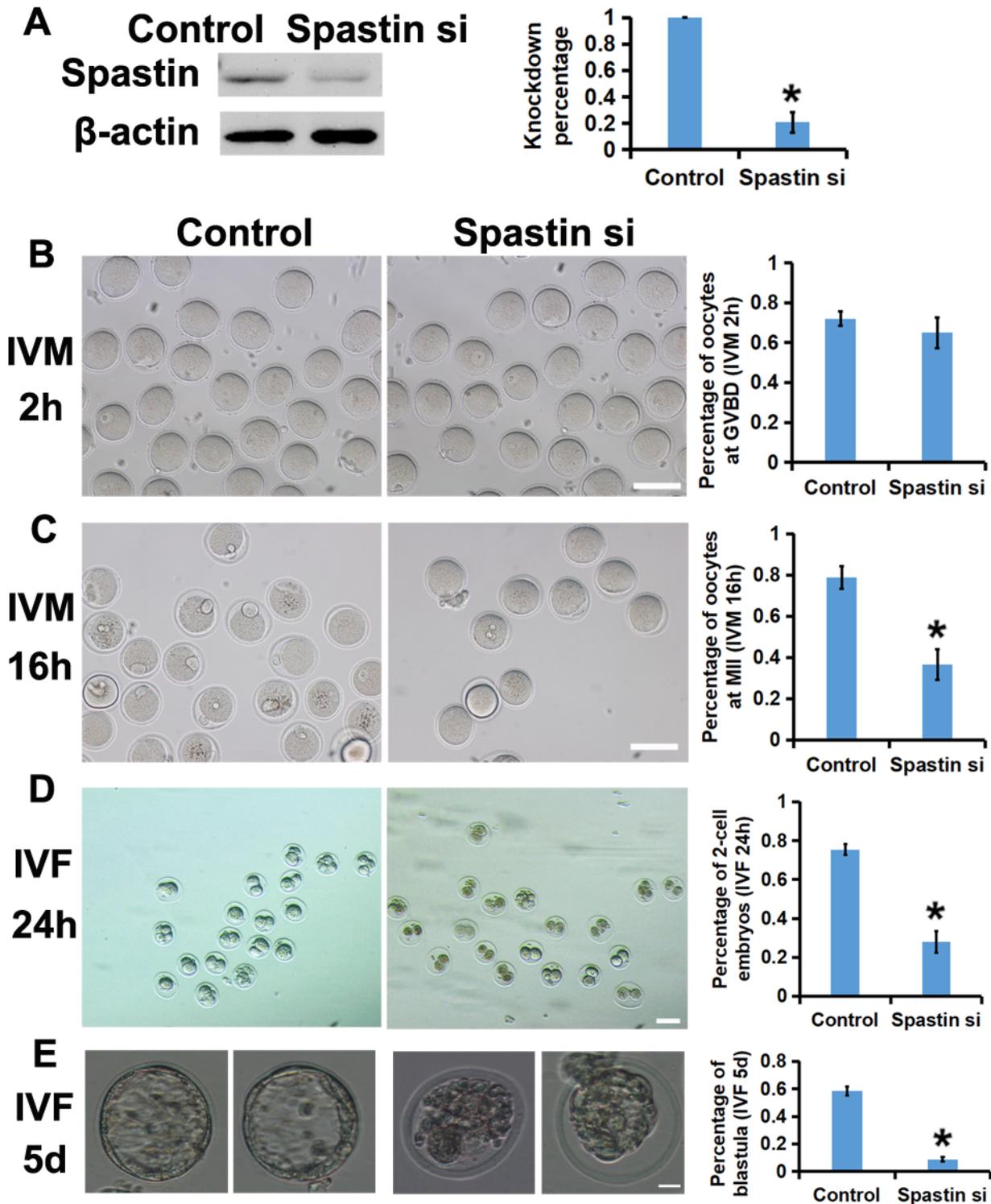


Figure 2

Spastin knockdown leads to severe maturation and fertilization abnormalities in oocytes. (A) Western blotting revealed that spastin was efficiently knocked down by specific siRNA. β -Actin was used as a control. (B) After 2 h of IVM, there was no significant difference in the percentage of GVBD oocytes between the spastin-knockdown and control groups. (C) After 16 h of IVM, there was a significant difference in the percentage of 1 Pb (first polar body) oocytes between the spastin-knockdown and

control groups. (D and E) IVF revealed that Spastin depletion induced a significant decrease in the two-cell embryo (D) and blastocyst rate (E). Scale bar, 100 μm . * $P < 0.05$ is considered to indicate statistically significant differences. IVF, in vitro fertilization.

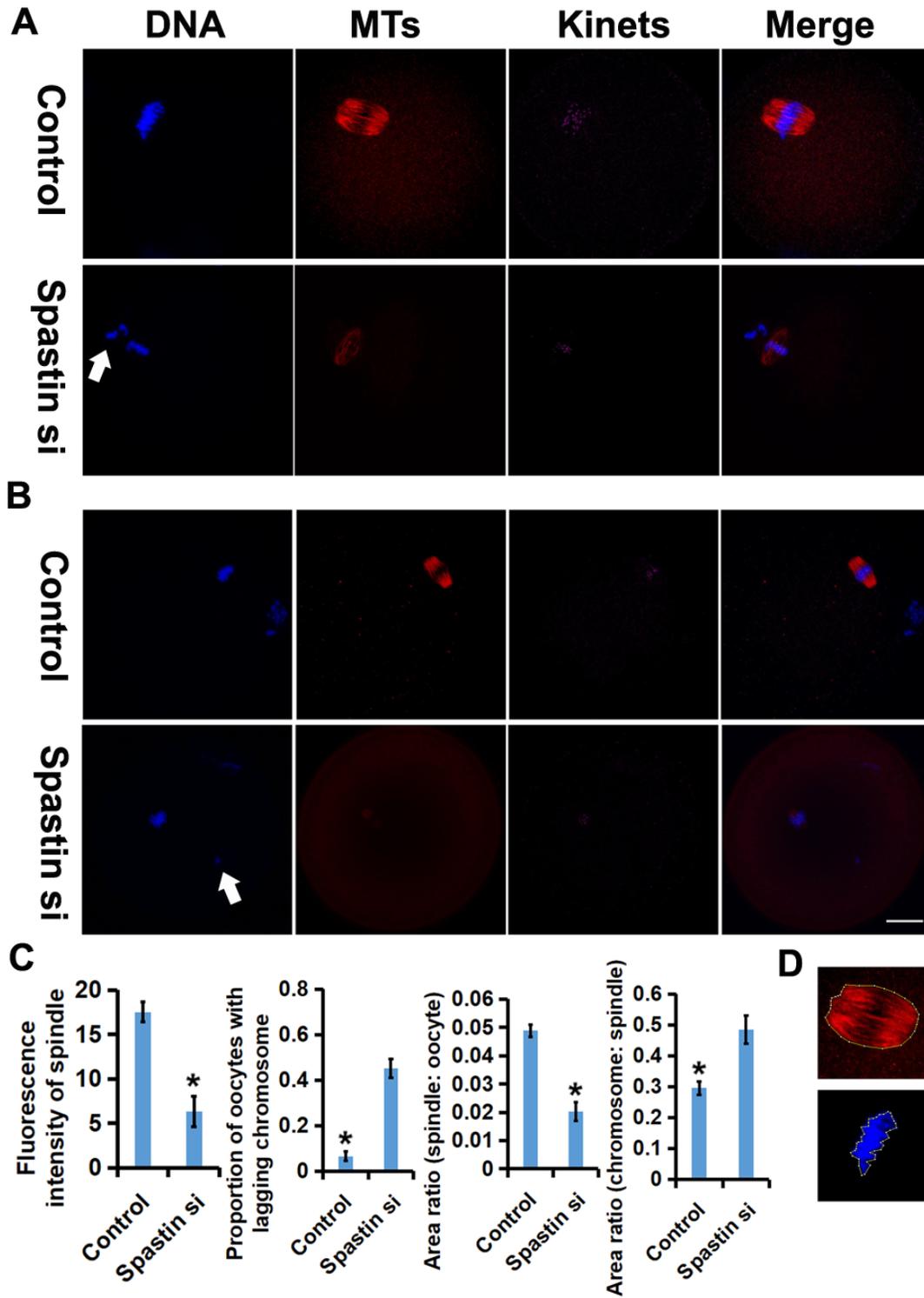


Figure 3

Spastin knockdown causes a significant decrease in the number and volume of spindle microtubules in MI and MII oocytes. (A and B) In MI (A) and MII (B) -stage oocytes, the fluorescence intensity of spindle

microtubule staining in spastin-deficient oocytes was significantly lower than that in the control group. In addition, in both MI (Fig.3A arrow) and MII (Fig.3B arrow) oocytes, more lagging chromosomes were found in spastin-knockdown oocytes. Tubulin is displayed in red, kinetochores in magenta, and DNA in blue. Scale bar, 20 μ m. (C and D) Measurement and statistics of fluorescence intensity of spindle, proportion of oocytes with lagging chromosome, area ratio (spindle: oocyte), area ratio (chromosome: spindle) are shown in Fig.3C respectively. The measurement method of spindle and chromosome area is shown in Fig.3D. *P<0.05 is considered to indicate statistically significant differences.

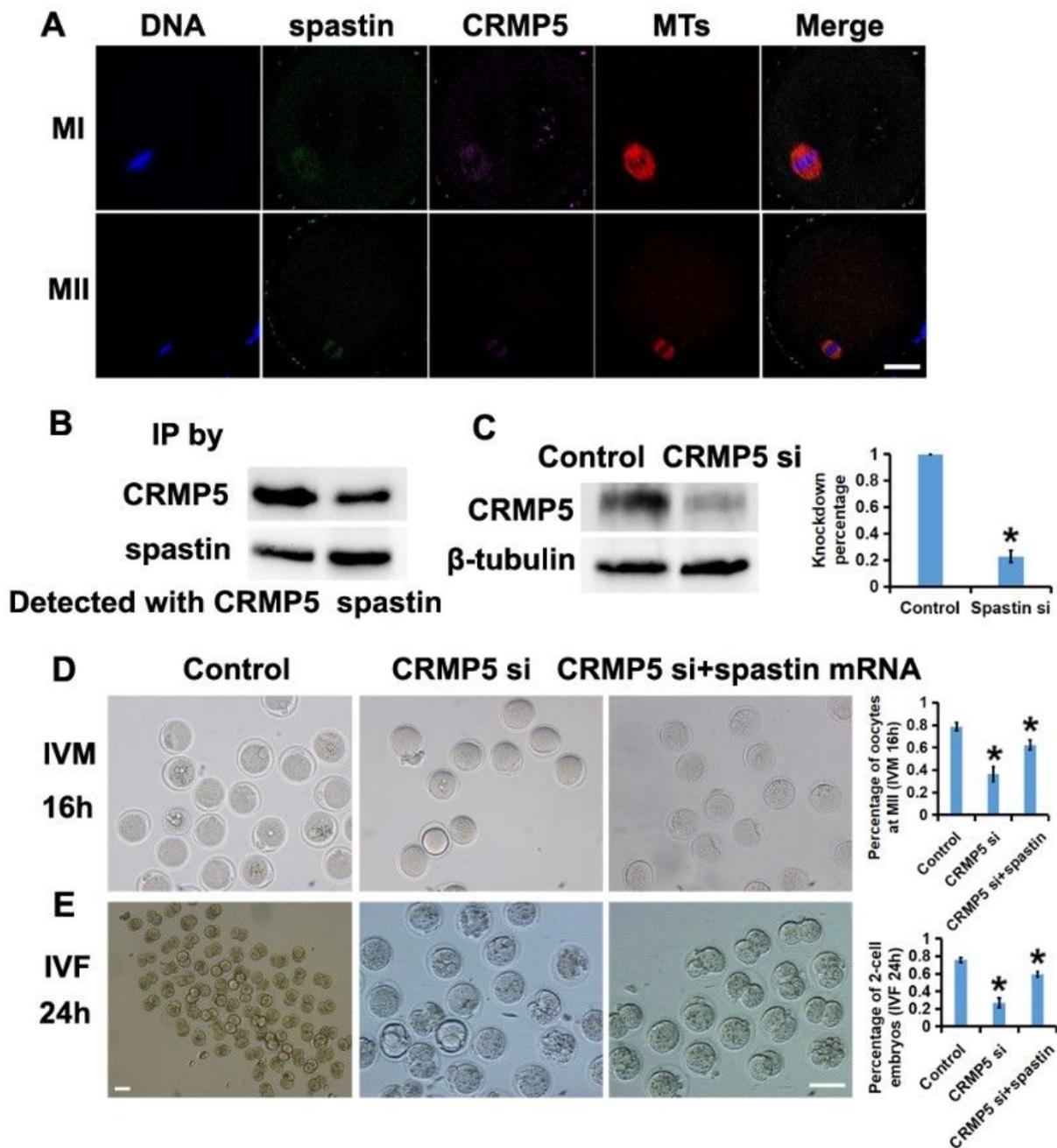


Figure 4

Exogenous spastin can rescue oocyte maturity disorder and abnormal fertilization ability caused by CRMP5 deletion. (A) Immunofluorescence showed that CRMP5 was distributed in spindle microtubules in oocyte and co-localized with spastin. (B) Co-IP results showed that CRMP5 and spastin could precipitate with each other. (C) Western blot showed that KL1 was able to be efficiently knocked down by specific siRNA. β -actin was used as a control. (D) After 16 h of IVM, there was a significant difference in the percentage of 1 Pb (first polar body) oocytes between the CRMP5-knockdown and control groups. The group of “CRMP5 siRNA+spastin mRNA” showed partial recovery of the percentage of 1 Pb oocytes. (E) IVF revealed that Spastin depletion induced a significant decrease in the two-cell embryo. The group of “CRMP5 siRNA+spastin mRNA” showed partial recovery of first two-cell embryo rate. Scale bar, 100 μ m. * $P < 0.05$ is considered to indicate statistically significant differences.

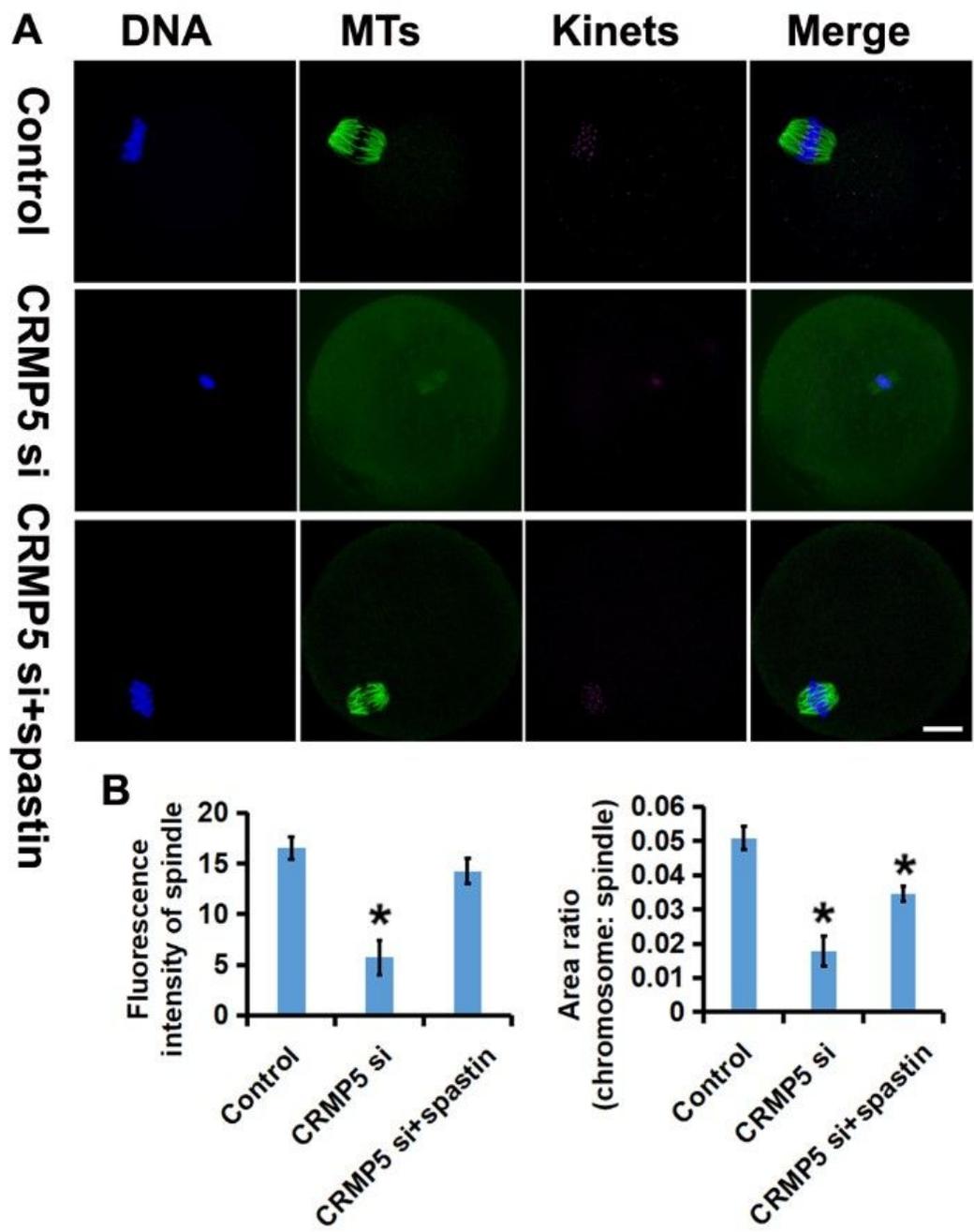


Figure 5

Exogenous spastin can rescue the decrease of spindle density and volume of oocyte caused by CRMP5 deletion. (A) Fluorescence intensity of spindle microtubule staining and spindle area in CRMP5-deficient oocytes was significantly lower than that in the control group. The group of “CRMP5 siRNA+spastin mRNA” showed partial recovery of above two indicators. (B) Measurement and statistics of fluorescence intensity of spindle, area ratio (chromosome: spindle) are shown in Fig.5B respectively. Scale bar, 20 μm . * $P < 0.05$ is considered to indicate statistically significant differences.

Supplementary Files

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