Preliminary investigation of the effects of an FCF inhibitor interference with Septin in the early stage embryos in mice

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Abstract. The objective of the present study was to investigate the effects of for chlorfenuron (FCF) interference with the septin protein on early stage embryos in mice. The 1-cell embryos were collected and divided into an FCF interference group and a control group. The FCF interference group was cultured in FCF media and the control group was cultured in dimethyl sulphoxide media at 37˚C with 5% CO_2 until the desired phase was achieved. Septin2 protein expression was detected using immunofluorescence and western blot analysis. Blastocyst α-tubulin was stained by immunofluorescence to observe the alterations in spindles and microtubules. The rate of early embryo development into blastocysts was significantly reduced following FCF treatment ($P<0.05$). In the control group, septin2 was observed with a confocal microscope; septin2 was expressed in embryos at all stages and mainly in the blastomeres from the 2-cell stage onwards, with the expression concentrated in the nuclei of the blastomeres as identified by strong fluorescence. In the FCF interference group, septin2 was weakly expressed in the nuclei of blastomeres at the 2- and 4-cell stages, and in the granulated blastomeres at the 4- and 8-cell stages. Expression was barely observed in and following the morula. Granulation was observed starting from the 4- and 8-cell stages. Compared with the control group, the FCF interference group exhibited irregular microtubules, abnormal spindle morphology and disordered chromosome arrangement in the blastocysts. The septin2 protein was expressed throughout the early stage embryo from the 2-cell stage to the blastocyst and localized in the nuclei of blastomeres. When the septin protein experienced interference by the FCF inhibitor, septin2 protein expression was reduced, which simultaneously resulted in abnormal embryonic development, uneven cytoplasmic division, various sizes and a reduced number of blastomeres, granulation in the blastomeres, disordered blastocyst microtubule distribution, spindle shape alterations and an abnormality of chromosome arrangement.

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Introduction

In recent decades, infertility and birth defects have continued to be common problems worldwide, and are becoming increasingly more serious (1-3). The fundamental reason for this lies in abnormality or failure during the generation and maturation of the germ cells or embryos, in which aneuploidy has been a stubborn genetic problem (1-3). According to statistics, 60% of the embryos in assisted reproduction have aneuploidy and are not easy to implant (1-3). Once implantation occurs, it is likely to be followed by early developmental stagnation and miscarriage; >50% of spontaneous abortions are caused by aneuploidy (1-3). The study of chromosomal ploidy screened by array comparative genetic hybridization method in the polar body/blastomere/trophoblast in the embryos of women aged >40 years old confirmed that 47.6% of aneuploidy was produced during the embryonic period (4). Therefore, the study of the pathogenesis of aneuploidy at the early stage embryo, reduction of aneuploidy occurrence and improvement of embryo quality has become the most important topics in reproductive science.

Septin is a large gene family and was initially identified in the mutant of *Saccharomyces cerevisiae* with abnormal cytoplasmic division (5). Presently, 13 septin genes have been demonstrated in the human body; these are classified into four categories based on the sequencing similarity of the genetic structure: SEPT2 (septin 1, 2, 4 and 5), SEPT3 (septin 3, 9, and 12), SEPT6 (septin 6, 8, 10, 11, and 14) and SEPT7 (6-8). Septin protein is filamentous and linked to actin, microtubules and cell membranes. Therefore, it is considered the fourth type of cytoskeleton (9). Initially, the septin family was recognized as a gene family associated with cytoplasmic division. However, with increased study of the septin protein, it has

been demonstrated that this type of gene has various functions in biology, especially in mammalian cells. Septin participates in multiple processes including cell membrane reconstruction, cell division, vesicle transportation and coordination of cell activities (10,11). Previous studies have demonstrated that septin2 is involved in the regulation of homologous chromosomal alignment and the process of meiosis during oocyte mitosis and that a knockdown of septin2/7 leads to abnormal separation and production of multinucleated cells (12,13).

For chlorfenuron [FCF; N-(2-chloro-4-pyridyl)-N0-phenylurea (C12H10ClN3O)] is a small molecule synthetic plant cytokinin, a growth enhancer and sweetening agent in agriculture that inhibits the metabolism of cytokinin dehydrogenase and increases fruit size (14). In non-plant eukaryotes, FCF can cause reversible ectopic septin structure (15). FCF has been demonstrated to interfere with the localization of yeast septins and to hinder the process of yeast budding (16), which also specifically affects the assembly, tissue and kinetic properties of mammalian septin proteins (17). A previous study confirm that the effect inhibited by FCF in mammalian cells has been demonstrated to phenocopy small interfering (si)RNA treatment (18). Furthermore, to study the role of septin2 in the meiosis process, Zhu *et al* (19) used FCF to treat oocytes and the results were the same as those where there was interference with siRNA. Importantly, oocyte viability was unaffected when oocytes cultured in the medium supplemented with FCF mimicked septin loss of function. Therefore, FCF provides a simpler and reliable alternative to study septin function.

The integrity of the spindle determines the efficiency of chromosome division; normal spindle formation is required for chromosome arrangement (20). To investigate whether septin can affect chromosomal arrangement and spindle assembly in early embryonic development, FCF chemical inhibitors were used to interfere with the septin protein; the septin2 protein was then used to confirm the reduction of septin. Localization and expression of septin2 was observed in each normal early stage of embryos using a confocal microscope and immunofluorescence. Similarly, the localization and expression of septin2 in embryos with intervention by FCF chemical inhibition was observed and then the effect on embryonic morphology and development was analyzed. The alterations of blastocysts, blastomeres, microtubules, spindles and chromosomes were compared between the two groups.

Materials and methods

Laboratory animals. Clean grade Kunming mice [20-30 g; with certificate SCXK (JI) 2013-01-003] were purchased from the Hebei Province Laboratory Animal Center (Shijiazhuang, China).

Reagents. M2 culture media, M16 culture media and FCF were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Paraformaldehyde (PFA) was purchased from Damao Chemical Reagent Factory (Tianjin, China). Triton X-100, Tween-20 and bovine serum albumin (BSA) were purchased from Beijing Solarbio Science & Technology Co., Ltd., (Beijing, China). Rabbit anti-septin2 antibody (cat. no. ab186020), fluorescein isothiocyanate (FITC) labeled goat anti-rabbit IgG (cat. no. ab6717), anti-α-tubulin-FITC (cat. no. ab64503), anti-GAPDH antibody (cat. no. ab8245) and horseradish peroxidase (HRP) labeled secondary anti-IgG antibody (cat. no. ab205718) were purchased from Abcam (Cambridge, UK). Hoechst 33258 dye and fluoro-gel with 1,4-diazabicyclo[2.2.2]octane; triethylenediamine were purchased from Sigma-Aldrich; Merck KGaA.

Equipment. A light stereoscopic microscope, incubator, 35 mm plastic petri dishes, 60 mm plastic petri dishes, surgical instruments and a confocal microscope were used.

Methods

Groups. All the animal procedures were approved by the Welfare Ethic Committee for Animal Experimentation of Hebei University (Baoding, China). A total of 30 female 8-week mice (20-30 g; housed at 4˚C, 60% humidity with a 12-h light/dark cycle and free access to food and water) were injected with pregnant mare serum gonadotropin (PMSG; 7.5 IU per mouse) on day 1, and then injected with human chorionic gonadotropin (hCG; 7.5 IU per mouse) on day 3 to induce superovulation. The female mice were placed together with 10-week male mice (n=30 male mice; 25-35 g; purchased from Hebei Province Laboratory Animal Center). Following the observation of copulatory plugs, the female mice were dissected and 1-cell stage embryos were collected. A total of 400 embryos with normal morphology were picked and divided into two groups, the FCF interference group and the control group; there were 200 embryos in each group. The FCF interference group was cultured in FCF culture media and the control group was cultured in dimethyl sulphoxide (DMSO) media at 37˚C with 5% CO_2 until reaching blastocysts. The number of blastocysts was then counted. Any remaining embryos other than the above 400 embryos were also divided evenly into two groups; the FCF interference group and the control group were cultured in the above two media, respectively, until the desired stage for the immunofluorescence staining of the septin protein was reached.

Preparation of reagents. Culture media containing FCF: The FCF was diluted using DMSO into a 50 mM stock solution. The stock solution was further diluted using M16 culture media to make a final concentration of 25 μM. DMSO culture media: The DMSO was diluted using M16 media to a final concentration of 25 μM.

Collection of embryos. On the second day following female and male mice being caged together, vaginal plugs were observed at the vaginas of the female mice. The presence of a vaginal plug indicated successful fertilization. Female mice with vaginal plugs were anesthetized using pentobarbital sodium and then euthanized by cervical dislocation. Each mouse was placed flat on the dissection plate and the abdominal skin was disinfected using 75% alcohol. The mouse peritoneal cavity was opened by an ophthalmic tweezer. The fallopian tube was removed and placed in a culture dish containing M2 *in vitro* buffer. Under the stereoscopic microscope, the bursa of the fallopian tube was pricked to release the fertilized eggs, which were surrounded by cumulus cells. Following digestion with hyaluronidase (1:1,000), the cumulus cells were repeatedly pipetted and removed by an egg pipette. Embryos at the 1-cell stage were picked up and

the abnormal embryos were discarded. The remaining normal embryos were washed using 37˚C M16 culture media 3-5 times to remove the residual hyaluronidase. The embryos were transferred by an egg pipette to 37˚C M16 culture media droplet, which was pre-equilibrated overnight (sealed with paraffin oil) with 20 embryos in each 50 μl droplet. The embryos were placed and cultured in the incubator at 37˚C with 5% CO_2 until the desired stage was achieved.

Culture of embryos. The embryos were placed in the incubator at 37˚C with 5% CO_2. The FCF interference group was cultured in FCF media until the blastocyst stage and the control group was cultured in DMSO media until the blastocyst stage for analysis of blastocyst rates, and α-tubulin antibody immunofluorescence staining in blastocysts. The remaining embryos were also cultured in the above two cultures for septin2 antibody immunofluorescence staining at all the embryonic stages.

Immunofluorescent staining. Embryos of each stage were placed in PBS droplets containing 1% PFA and 0.25% Triton X-100 for fixation and permeabilization for 1 h at 4˚C. They were then washed with PBS containing 1% BSA 3 times, the embryos were blocked in PBS containing 1% BSA for 1 h at 4˚C, diluted 1:25 with septin2/α-tubulin antibodies and incubated at 4˚C overnight. After 20-24 h, the embryos were washed with PBS containing 0.1% Tween 20 and 0.01% Triton X-100 5 times and incubated in FITC labeled secondary anti-IgG antibody (1:100) at room temperature for 1 h. Subsequently, the embryos were washed 5 times and the nuclei were stained with Hoechst 33258 (10 μg/ml in PBS) for 10 min at room temperature. Following eluting 5 times, the embryos were transferred to a glass slide and quenched with fluorescence quenching mounting media. The protein and chromosomes were detected by a laser scanning confocal microscopy.

Western blotting. Given that the number of mouse embryos was too large and the rate of embryo cleavage is different, the mouse embryos that were cultured at the same time (including the 2-cell embryo group, the 4- to 8-cell embryo group, the mulberry embryo group and the blastocyst group) were collected (every 200 embryos were a sample), and the embryos were boiled in SDS loading buffer (4% SDS, 10% mercaptoethanol, 2% glycerinum, bromophenol blue 0.2 mg/ml) for 5 min. The proteins (200 embryos/sample) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Then, the membranes were blocked by TBS with 0.1% Tween-20 (TBST) solution containing 5% skim milk powder for 2 h at 4˚C, diluted 1:500 with septin2/α-tubulin and incubated at 4˚C overnight. Then, the membranes were washed with TBST and incubated in HRP-labeled secondary anti-IgG antibody (1:1,000) at 37˚C for 1 h. Finally, the membranes were coated with immobilon western HRP (EMD Millipore, Billerica, MA, USA) exposed by an enhanced chemiluminescence detection system (Bio-Rad Chemic-Doc XRS+; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All of the experiments were repeated three times. SPSS19.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis of experimental data. The blastocyst rates of the two groups were compared using the chi-square test. P<0.05 was considered to indicate a statistically significant difference.

Table I. Effects of the addition of FCF on the percentage of embryos that reach the blastocyst stage.

Groups	Embryo (n)	Blastocyst (n)	Percentage of blastocysts (%)
FCF interference group	200	63	31.5[a]
Control group	200	151	75.5

χ^2=77.821; [a]P<0.001 vs. the control group. FCF, Forchlorfenuron.

Results

Effects of FCF on blastocyst rates. Following treatment by FCF, the number of blastocysts was significantly reduced compared with the control group. The difference of the blastocyst rates between the two groups was significant (P<0.001; Table I).

Expression of α-tubulin in mouse blastocysts. In the control group, α-tubulin was present in the cytoplasm of the blastomeres and on the spindles. The blastomeres were distributed in a regular pattern and a substantial number of consistent size were observed, the chromosomes were regularly arranged on the equatorial plate of the spindle. The spindle was in good condition (as presented in Fig. 1A and B). In the FCF interference group, α-tubulin was irregularly located. In addition, blastomeres were markedly decreased in number, with various sizes and shapes. The boundaries of the blastomeres were not clear, the spindles became long and wide. The chromosomes were not gathered on the equatorial plate but were arranged in an irregular pattern (as presented in Fig. 1C and D).

Expression of Septin2 in each stage of the mouse embryos. Septin2 protein expression was observed by confocal microscopy in each stage of the mouse embryo. As presented in Fig. 2, in the control group, the cells exhibited a good morphology with clear nuclear materials. Septin2 protein was mainly distributed on the nuclei of blastomeres at the 2-cell stage (Fig. 2A), the 4-cell stage (Fig. 2B) and the 8-cell stage (Fig. 2C), which was revealed by strong fluorescence. When the morula and subsequent stages were reached, the connection between blastomeres became tighter. Septin2 protein was localized on the nuclei, but the boundary of the blastomeres was less clear than in previous stages and the immunofluorescence strength also became weak (Fig. 2D and E). From the blastocyst stage, the blastomeres began to gather to the side of the trophoblasts and a cavity was gradually formed in the center. Septin2 was mainly distributed in the nuclei.

In the FCF interference group, septin2 expression was markedly reduced. In the embryos at the 2-cell stage, septin2 was slightly expressed on the nuclei of blastomeres. The two blastomeres were of different sizes with one bigger and one smaller (Fig. 3A). Septin2 was weakly expressed on one of the blastomeres at the 4-cell stage, where the blastomeres exhibited

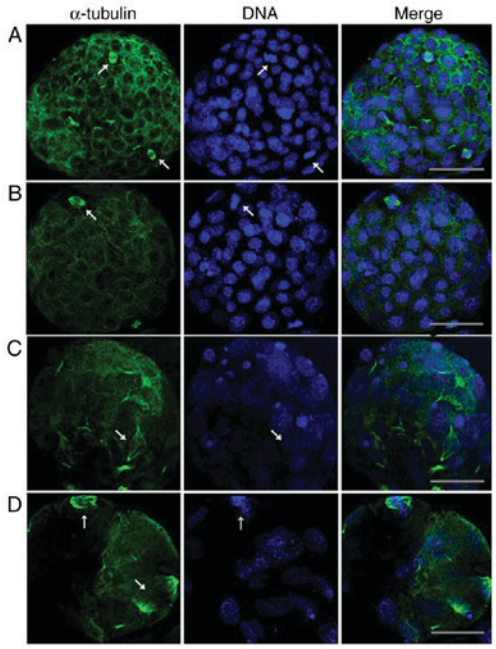

Figure 1. Location of α-tubulin and DNA in two groups of blastocysts. In the figure, green fluorescence represents α-tubulin and blue fluorescence represents DNA. (A and B) Two sets of representative images of the control group, the arrow marks the spindle with a good shape and uniform size, and the DNA was regularly arranged on the equatorial plate. (C and D) Two sets of representative images of the FCF interference group, the arrows mark with various sizes and shapes of the spindles, and the chromosomes with irregular arrangement. Scale bar=20 μm. FCF, Forchlorfenuron.

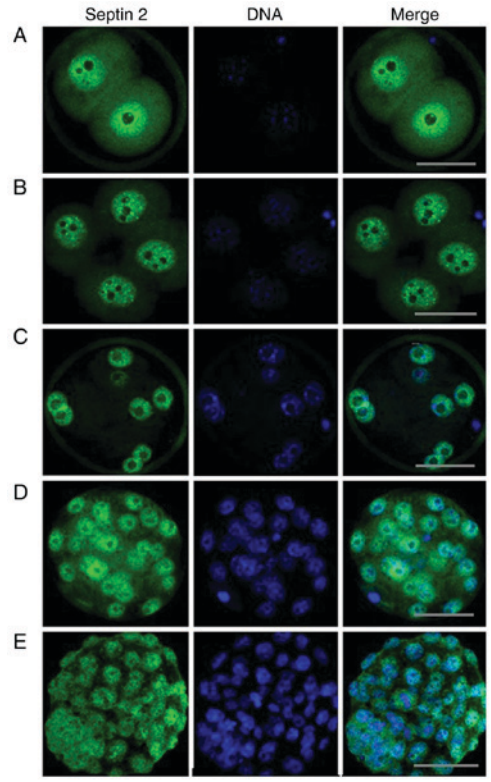

Figure 2. Localization of septin2 protein in each stage of the embryos in the control group. Septin2 protein localization of the (A) 2-cell embryo, (B) 4-cell embryo, (C) 8-cell embryo, (D) morula embryo and the (E) blastocyst of the control group. Septin2 was expressed in the embryos of all of the stages and mainly concentrated in the nuclei of the blastomeres, which was revealed by strong fluorescence, and the size and number of cleavage balls were normal. Scale bar=20 μm.

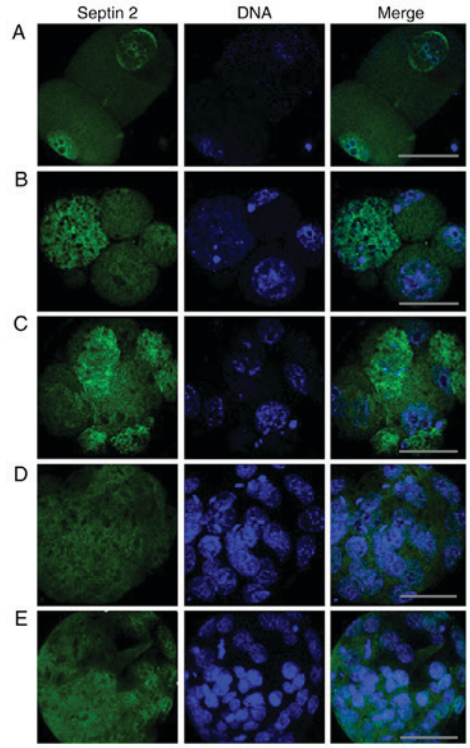

Figure 3. Localization of the septin2 protein in each stage of the embryos in the FCF interference group. Septin2 protein localization of the (A) 2-cell embryo, (B) 4-cell embryo, (C) 8-cell embryo, (D) morula embryo and (E) blastocyst of the FCF interference group. Septin2 was weakly expressed in the nuclei of the blastomeres at the 2-and 4-cell stages and in the granulated blastomeres at 4- and 8-cell stages. Expression was barely observed in and regarding the morula stage. Granulation was observed starting from the 4- and 8-cell stages and the morphology and size of the cleavage balls were abnormal. Scale bar=20 μm. FCF, Forchlorfenuron.

different sizes and granulation occurred in certain blastomeres (Fig. 3B). At the 8-cell stage, granulation continued and septin2 was weakly expressed in the cytoplasm of the granulated blastomeres. The nuclei of the blastomeres were of an abnormal morphology (Fig. 3C). From morula to blastocysts, septin2 was barely expressed. The number and the size of the blastomeres were irregular compared with the control group (Fig. 3D and E). Western blot experiment result demonstrated that septin2 was marginally expressed at the 2-cell stage and subsequent stages demonstrated almost no expression (Fig. 4). In addition, it was revealed that the expression of the septin2 protein was similar to that of immunofluorescence, septin2 was expressed in embryos of all the stages (Fig. 4).

Discussion

As a GTP binding protein, septin can form septin fibrils to participate in cytoplasmic division through the GTP binding site or interaction with the C-terminal helix domain. Septin proteins in mammals are localized with microtubules and microfilaments. As the two most important cytoskeletons in cytoplasmic division, microfilaments and microtubules regulate the assembly of septin fibrils. Septin can regulate the accumulation of chromosomes to the equatorial plate and the correct separation at the anaphase of mitosis. In this environment, the SEPT2/6/7 complex is very important for

Figure 4. Expression of septin2 protein in each phase of the embryos in the two groups. The left side was septin 2 and GAPDH protein expression of the control group, and the right side was septin 2 and GAPDH protein expression of the FCF interference group; septin2 was expressed in embryos of all the stages. The (Lane A) 2-cell stage embryo, (Lane B) 4- to 8-cell stage embryo, (Lane C) morula and (Lane D) blastocyst, and septin2 were expressed marginally at the 2-cell stage and subsequent stages exhibited almost no expression. FCF, Forchlorfenuron.

the supplementation of motor protein centromere-associated protein E (CENP-E) at the mitosis checkpoints. Knockdown of the septin protein may reduce the number of chromosomes accumulated at the equatorial plate and lead to abnormal chromosomal separation and spindle expansion. This defect is associated with a lack of motor protein CENP-E, which moves to the positive end of the microtubules. It has been demonstrated that in the epithelial cells, interference with septin2 results in the loss of glutamylated microtubules and disordered chromosome arrangement (21-23).

In addition to regulating physiological functions, including cell cycle, membrane reconstruction and targeted transport of vesicles (24-26), septin protein is associated with certain human diseases including neurological diseases, tumors, infections and infertility (27-30). Aneuploidy and multinucleated phenomena of human early embryos are similar to the development of tumor cells. A number of studies have demonstrated that upregulation or downregulation of septin protein expression occurs with cancerous alterations in the blood and a number of solid tumors (31-33). Septin participates in the mitotic process and therefore potentially affects tumorigenesis. A recent study revealed that overexpression of septin2 or of ectopic aniline and septin9 can lead to aneuploidy, centrosome amplification and multipolar mitosis, and therefore affect cytoplasmic division. This occurs frequently in tumor cells (34). To analyze the effect of septin on embryonic development and division, FCF was used to intervene in early embryos as experimental groups and observed the expression and localization of the septin 2 protein in the early embryos of mice to verify the septin protein reduction situation. It was observed that septin2 was expressed in all 2-cell, 4-cell, 8-cell, morula and blastocyst stages of embryos, which were cultured in DMSO media. The expression was mainly observed on the nuclei of blastomeres and identified by strong fluorescence. In embryos cultured in FCF media, septin2 expression was reduced. A small amount of expression was observed on the nuclei of the blastomeres at the 2-cell stage, where blastomeres varied in sizes. Certain blastomeres at the 4-cell and 8-cell stage embryos exhibited a high level of granulation. Slight expression of septin2 was only observed in granulated blastomere cytoplasm and the nuclear morphology was abnormal in the blastomeres. In the FCF interference group septin2 was barely expressed in the morula and blastocysts where the number and size of the blastomeres were irregular compared with the control group. These results indicate that FCF inhibited septin expression and reduced septin levels, which leads to abnormal embryonic development and is more specifically presented as uneven cytoplasmic division, various sizes and a reduced number of blastomeres, disordered chromosomal arrangement and granulation in the embryos. This suggests that septin serves an important role in mouse early stage embryonic development and division.

Considering that FCF has been widely used to inhibit septin function and that its effect on cells were similar to those induced by a microinjection of siRNA (even while the cellular viability was unaffected), FCF was used to interfere with septin to study the function of the protein in the early embryo. In the present study, α-tubulin was stained in the developing embryos until the blastocyst stage in the FCF interference and control groups, and the effect on embryonic development and division was evaluated by observing microtubule distribution, spindle assembly and chromosomal arrangement. It was demonstrated that following FCF treatment, the embryos exhibited irregular distribution of microtubules, abnormal spindle morphology and chromosomal arrangement, and a reduced number of blastomeres compared with the DMSO control group. In addition, the blastocyst rate in the FCF-treated group was significantly decreased compared with in the control group, which further indicated that septin served an important role in early stage embryo development and division and participated in spindle assembly and chromosome arrangement in early embryo mitosis. The mechanism remains to be determined in future studies and will be the next study that will be conducted. In subsequent research, the authors aim to continue to investigate other members of the septin family to study the function of the septin protein in early mouse embryos.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SG was responsible for data analysis, and was a major contributor in writing the manuscript. YW and JZ completed embryo acquisition and culture as well as the immunofluorescence experiments. YT conducted the western blotting experiments. SW and ZG analyzed and interpreted the data regarding early embryo development and Septin protein levels, and were also responsible for the project and manuscript design.

Ethics approval and consent to participate

All of the animal procedures were approved by the Welfare Ethic Committee for Animal Experimentation of Hebei University (Baoding, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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