

# Geminin deletion in mouse oocytes results in impaired embryo development and reduced fertility

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**ABSTRACT** Geminin controls proper centrosome duplication, cell division, and differentiation. We investigated the function of geminin in oogenesis, fertilization, and early embryo development by deleting the geminin gene in oocytes from the primordial follicle stage. Oocyte-specific disruption of geminin results in low fertility in mice. Even though there was no evident anomaly of oogenesis, oocyte meiotic maturation, natural ovulation, or fertilization, early embryo development and implantation were impaired. The fertilized eggs derived from mutant mice showed developmental delay, and many were blocked at the late zygote stage. Cdt1 protein was decreased, whereas Chk1 and H2AX phosphorylation was increased, in fertilized eggs after geminin depletion. Our results suggest that disruption of maternal geminin may decrease Cdt1 expression and cause DNA rereplication, which then activates the cell cycle checkpoint and DNA damage repair and thus impairs early embryo development.

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## INTRODUCTION

Geminin (Gmn) was identified in an expression cloning screen in *Xenopus* embryos (Kroll *et al.*, 1998) and was found to inhibit DNA replication and become degraded during the mitotic phase of the cell cycle (McGarry and Kirschner, 1998). As an unstable regulatory protein, geminin has two different molecular functions, which control patterns of cell division and differentiation (Luo and Kessel, 2004; Seo and Kroll, 2006; Yoshida, 2007). The main function of geminin is to regulate DNA replication. Geminin binds to and inhibits the essential replication factor Cdt1 to limit the extent of DNA replication to one round per cell cycle (McGarry and Kirschner, 1998; Wohlschlegel *et al.*, 2000).

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Abbreviations used: Gmn, geminin; GV, germinal vesicle; GVBD, germinal vesicle breakdown; IVC, in vitro culture; IVF, in vitro fertilization; MI, metaphase of first meiosis; MII, metaphase of second meiosis.

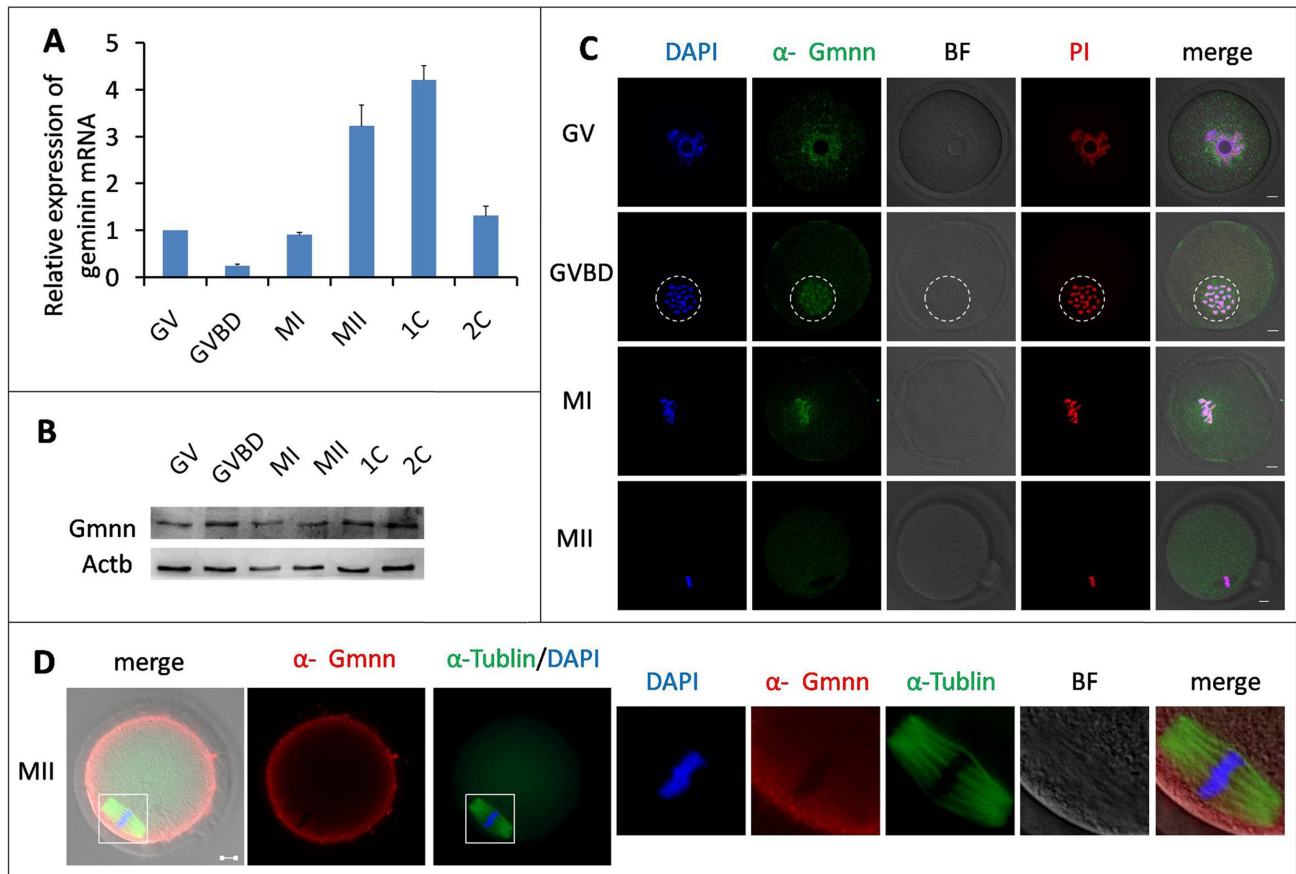
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Geminin also affects cell differentiation by binding and inhibiting transcription factors and chromatin-remodeling proteins, such as members of the Homeobox family, Brg1, Six3, and Scmh1 (Del Bene *et al.*, 2004; Luo and Kessel, 2004; Seo *et al.*, 2005; Zhou *et al.*, 2012). During development of the nervous system, geminin controls the transition from proliferating precursor to differentiated postmitotic neurons by modulating interactions between SWI/SNF and basic helix-loop-helix transcription factors, which are critical for neurogenesis (Seo and Kroll, 2006). In early embryo development, geminin deficiency quickly leads to a loss of pluripotency and differentiation into the mesendodermal direction, with high Oct4/low Sox2 levels (Gonzalez *et al.*, 2006; Tabrizi *et al.*, 2013).

In mitosis, geminin is partially localized to the centrosome and plays a role in proper centrosome duplication (Lu *et al.*, 2009). A fraction of geminin is localized to the centrosome, and the centrosomal localization of geminin is Arp1 mediated and dynein-dynactin dependent. The coiled-coil motif of geminin is required for its targeting to the centrosome and inhibition of centrosome duplication.

In meiosis, depletion of geminin in *Xenopus* immature oocytes leads to a decrease of Cdt1 protein level during maturation and after activation of these oocytes. Geminin also acts as a stabilizer of Cdt1, promoting its accumulation for the early division cycles of the



**FIGURE 1:** Expression and localization of geminin in mouse oocytes. The mRNA (A) and protein (B) expression of geminin during meiotic maturation of mouse oocytes and early development of mouse embryos. Oocytes were cultured for 0, 4, 8, and 12 h, corresponding to germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase of first meiosis (MI), and metaphase of second meiosis (MII) stages, respectively. Here 1C and 2C indicate one- and two-cell embryos, respectively. Error bars indicate the SEM. The experiments were repeated three times. (C) Confocal micrographs showing subcellular localization of immunostained geminin (green) and DNA (DAPI/blue and PI/red) in mouse oocytes at GV, GVBD, MI, and MII stages. BF, bright-field image. Bar, 10  $\mu$ m. (D) Confocal micrographs showing localization of immunostained geminin (red),  $\alpha$ -tubulin (green), and DNA (DAPI/blue) in mouse oocytes at MII stages. Bar, 10  $\mu$ m.

embryo (Narasimhachar and Coue, 2009). Although geminin is required for mitotic proliferation of spermatogonia, deleting geminin from spermatocytes does not disrupt meiosis or the differentiation of spermatids into mature spermatozoa (Barry *et al.*, 2012).

In this study, by using the *Gdf9-Cre/loxP* system, we selectively deleted geminin in mouse oocytes to investigate its roles in oogenesis and embryo development. We found that geminin deletion did not disrupt oocyte development, meiotic maturation, ovulation, or subsequent fertilization, but did result in zygote developmental delay by activating DNA rereplication and the cell cycle checkpoint of the first cell cycle.

## RESULTS

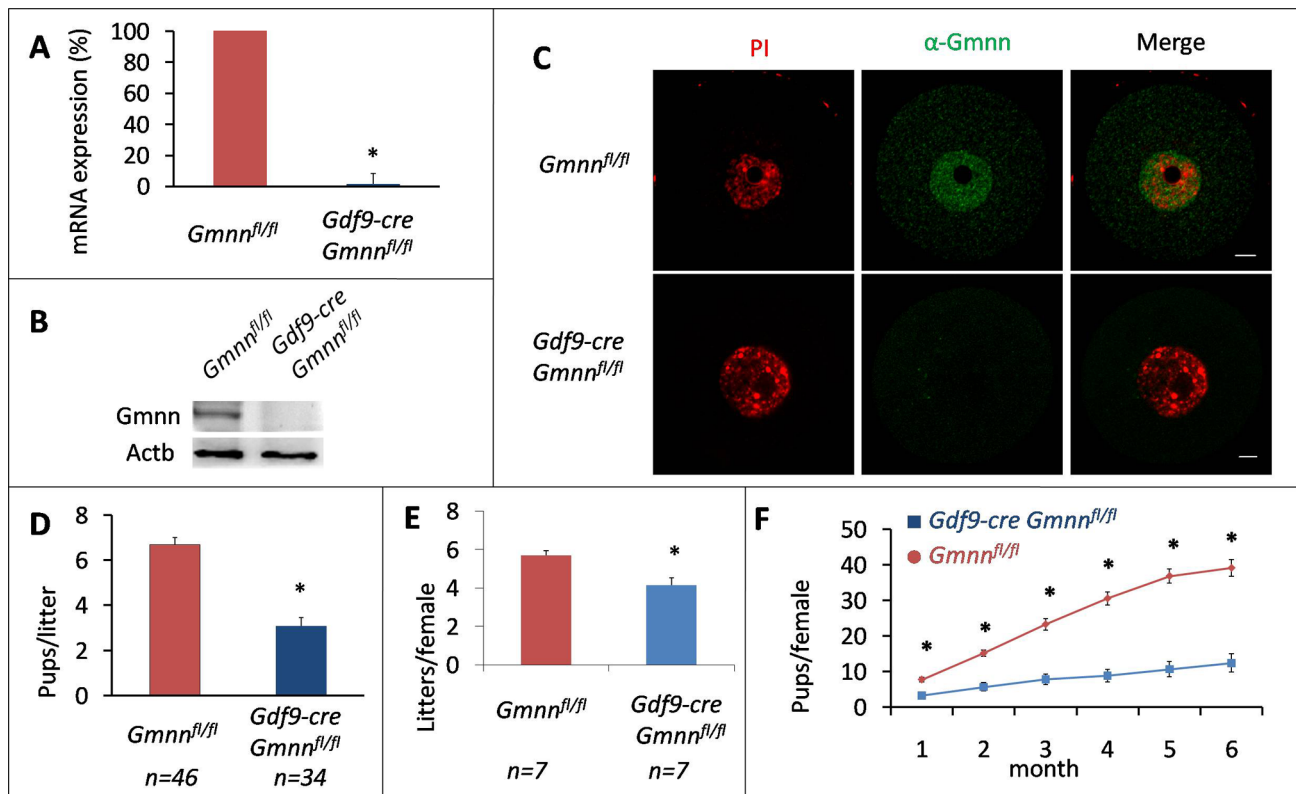
### The expression of geminin in oocytes and early embryos

We first examined the expression and distribution of geminin in oocytes. By quantitative real-time PCR and Western blot analysis, we detected geminin expression during all stages of oocyte maturation and early embryo development (Figure 1, A and B). Oocytes at the germinal vesicle (GV) stage, the germinal vesicle breakdown (GVBD) stage, metaphase of first meiosis (MI), and metaphase of second meiosis (MII) were selected for immunofluorescence staining. In the

GV oocytes, geminin localized in the nucleus, and after GVBD, geminin still localized to the nuclear area (indicated by white dashed circle in Figure 1C). At the MI stage, geminin colocalized with DNA; it did not display a specific localization after reaching the MII stage (Figure 1, C and D).

### Oocyte-specific disruption of the geminin gene

Mutant mice with oocyte-specific disruption of the geminin gene, in which exons 5–7 of the geminin gene were targeted in oocytes (Shinnick *et al.*, 2010), were generated by crossing *Gmnn<sup>fl/fl</sup>* mice with transgenic mice expressing *Gdf9* promoter-mediated Cre recombinase (Lan *et al.*, 2004). In *Gdf9-Cre* mice, Cre recombinase expression starts at day 3 after birth and is exclusively expressed in oocytes of primordial follicles and in later developmental stages (Lan *et al.*, 2004; Sun *et al.*, 2008). Quantitative real time-PCR and Western blot analysis showed that in *Gdf9-cre Gmnn<sup>fl/fl</sup>* mouse oocytes, the geminin mRNA and protein were depleted significantly (Figure 2, A and B). The geminin mRNA in *Gdf9-cre Gmnn<sup>fl/fl</sup>* mouse oocytes was only  $1.45 \pm 0.98\%$  (SEM,  $n = 3$ ) of that for the *Gmnn<sup>fl/fl</sup>* group. Immunofluorescence analysis of oocytes from *Gdf9-cre Gmnn<sup>fl/fl</sup>* mice revealed loss of geminin localization in the nucleus



**FIGURE 2:** Knockout efficiency of geminin and fertility analysis of *Gdf9-cre Gmnn<sup>fl/fl</sup>* female mice. (A) mRNA expression of geminin in *Gdf9-cre Gmnn<sup>fl/fl</sup>* mouse oocytes. Expression is displayed relative to mouse actb. Error bars indicate the SEM. The experiments were repeated three times. (B) Geminin protein depletion in *Gdf9-cre Gmnn<sup>fl/fl</sup>* mouse oocytes as detected by Western blot analysis. actb was used as a loading control. (C) Immunostained geminin (green) and DNA (red) in *Gdf9-cre Gmnn<sup>fl/fl</sup>* mouse oocytes. Bar, 10  $\mu$ m. (D) Reduced average number of pups per litter in the *Gdf9-cre Gmnn<sup>fl/fl</sup>* female group. (E) Reduced number of litters per female at 6 mo. (F) Reduced number of pups per female at 6 mo. \* $p < 0.01$ .

(Figure 2C). We thus confirmed that geminin was selectively knocked out and its protein depleted in *Gdf9-cre Gmnn<sup>fl/fl</sup>* mouse oocytes.

### Oocyte-specific deletion of geminin results in low fertility

Breeding assays showed that female *Gdf9-cre Gmnn<sup>fl/fl</sup>* mice were evidently subfertile, and the average pups number per litter was  $3.09 \pm 0.3$  (Figure 2D), which was significantly lower than in the control group ( $6.70 \pm 0.375$  pups,  $p < 0.01$ ). Within 6 mo, the average numbers of pups and litters of *Gdf9-cre Gmnn<sup>fl/fl</sup>* females were significantly lower than those of the control group ( $p < 0.01$ ; Figure 2, E and F). The average litter number of *Gdf9-cre Gmnn<sup>fl/fl</sup>* females was only  $4.14 \pm 0.38$ , whereas that of the control group was  $5.71 \pm 0.23$  (Figure 2E). These data suggested that specific knockout of the geminin gene in oocytes could cause a significant decrease of fertility in mice.

### Oocyte maturation and fertilization are normal in mutant mice

To find the cause of low fertility in mutant mice, we examined the ovary/follicle development and oocyte maturation. Bilateral ovarian weight of the *Gdf9-cre Gmnn<sup>fl/fl</sup>* mice and *Gmnn<sup>fl/fl</sup>* mice showed no significant difference ( $5.23 \pm 0.38$  and  $5.83 \pm 0.47$  mg, respectively; Figure 3A). Histological analysis of the ovary showed that follicular development was normal (Figure 3B).

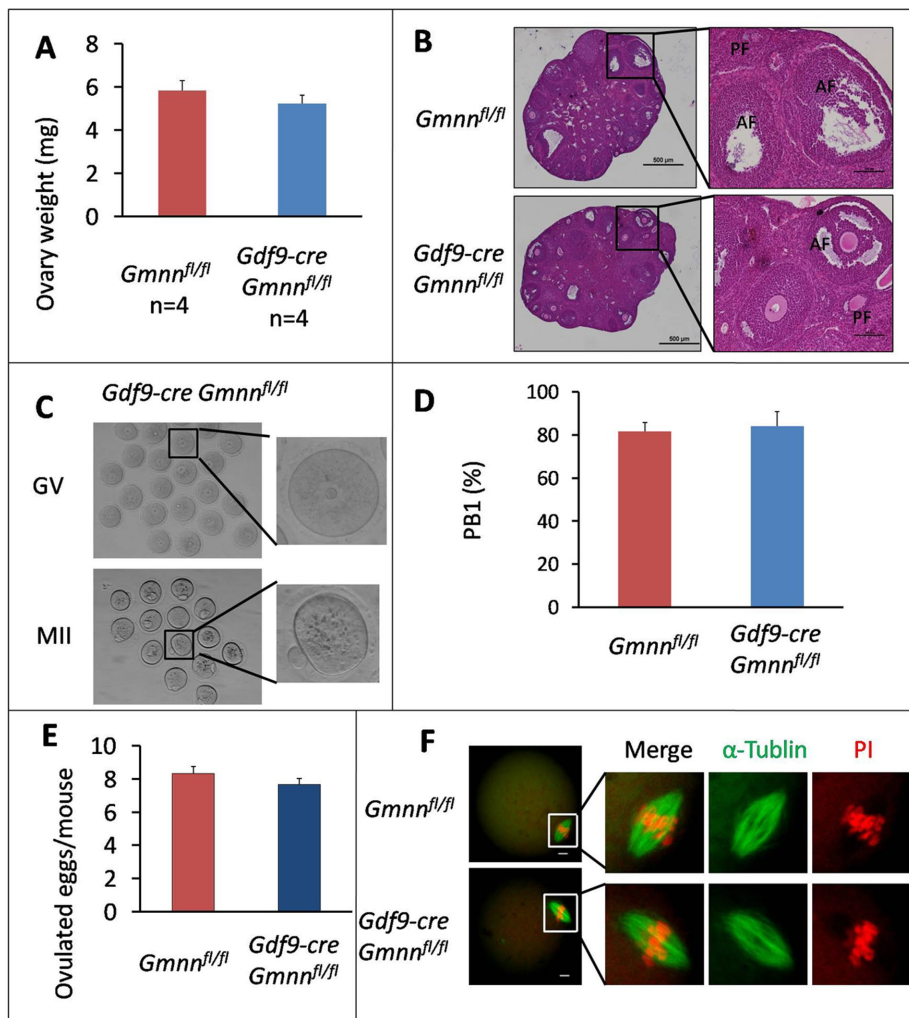
The GV stage oocytes isolated from ovaries of 6- to 9-wk-old female *Gdf9-cre Gmnn<sup>fl/fl</sup>* mice showed no abnormal appearance

(Figure 3C). They could develop in vitro to the MII stage, and the first polar body rate showed no significant difference compared with the control group (Figure 3D). Natural ovulation detection revealed that the ovulation of mutant mice displayed no significant difference compared with the control group. The *Gdf9-cre Gmnn<sup>fl/fl</sup>* mice ovulated an average of  $7.67 \pm 0.47$  eggs, whereas the *Gmnn<sup>fl/fl</sup>* mice ovulated an average of  $8.33 \pm 0.45$  eggs (Figure 3E). Both in vitro- and in vivo-matured oocytes displayed normal spindle morphology and chromosome alignment (Figure 3, C and F).

The natural ovulated eggs were all fertilized and appeared normal, with male and female pronuclei. By using 5-bromo-deoxyuridine (BrdU) labeling, we also confirmed that DNA replication occurred in the mutant fertilized eggs (as shown later in Figure 5A). These results indicated that the deletion of the geminin gene had no significant effect on oocyte development/maturation, ovulation, and fertilization in *Gdf9-cre Gmnn<sup>fl/fl</sup>* mice.

### The development of mutant fertilized eggs is delayed

By embryo implantation site detection (Figure 4, A and B), we found that the number of implantation sites of *Gdf9-cre Gmnn<sup>fl/fl</sup>* females was significantly lower than that of the *Gmnn<sup>fl/fl</sup>* females ( $2.33 \pm 1.3$  vs.  $9 \pm 0.8$ ,  $p < 0.01$ ) on day 5. The results indicated that oocyte-specific disruption of geminin might influence preimplantation embryo development. We further investigated the two-cell embryo development in vitro after insemination. Only  $22.7 \pm 4.52\%$  of the fertilized eggs in the experimental group developed to two-cell



**FIGURE 3:** The development of follicles and natural ovulation in *Gdf9-cre Gmnn<sup>fl/fl</sup>* female mice. (A) The average weight of ovaries from 8-wk-old females. (B) Hematoxylin and eosin staining of representative ovaries at 4 $\times$  (bar, 500  $\mu$ m) and 40 $\times$  (bar, 100  $\mu$ m) magnification. AF, antral follicle; PF, primary follicle. (C) The bright-field images of mutant GV and MII oocytes at 20 $\times$  magnification. (D) The first polar body rate for *Gmnn<sup>fl/fl</sup>* and *Gdf9-cre Gmnn<sup>fl/fl</sup>* female group. Error bars indicate the SEM. The experiments were repeated three times. (E) The average number of ovulated eggs. Four female mice with plugs were examined for each group. Error bars indicate the SEM. (F) Confocal micrographs showing  $\alpha$ -tubulin (green) in MII eggs. Bar, 10  $\mu$ m.

embryos compared with  $95.8 \pm 1.23\%$  in the *Gmnn<sup>fl/fl</sup>* group at 2 d after mating ( $p < 0.01$ ; Figure 4C).

Most arrested embryos obtained from *Gdf9-cre Gmnn<sup>fl/fl</sup>* mice were blocked at the zygote stage. A representative result is shown in Figure 4D. The male and female pronuclei of the blocked zygotes did not break down, which indicates that these fertilized eggs did not enter metaphase of the first mitosis. When fertilized eggs from natural ovulation were cultured in vitro, we found that development of most fertilized eggs from *Gdf9-cre Gmnn<sup>fl/fl</sup>* mice was delayed (Figure 4E).

### Geminin depletion causes DNA rereplication in mutant fertilized eggs

Rereplication can be induced by geminin depletion in *Xenopus* (McGarry, 2002; Kerns et al., 2007, 2012) and in U2OS human osteosarcoma cells (Klotz-Noack et al., 2012). By using BrdU labeling, we examined whether DNA rereplication occurred in the mutant

fertilized eggs. DNA replication commonly occurs at 6–8 h of in vitro fertilization (IVF) in fertilized oocytes. Fertilized eggs at 6 or 9 h of IVF were incubated in human tubal fluid (HTF) medium supplemented with BrdU and then collected and immunostained at 14 h of IVF. There was almost no BrdU labeling signal in fertilized eggs from *Gmnn<sup>fl/fl</sup>* mice, which indicated the absence of DNA rereplication. In contrast, a distinct signal was detected in the fertilized eggs obtained from *Gdf9-cre Gmnn<sup>fl/fl</sup>* mice. The results indicated that DNA rereplication occurred in mutant fertilized eggs.

### Geminin depletion causes decreased Cdt1 expression and increased Chk1 and H2AX phosphorylation in mutant fertilized eggs

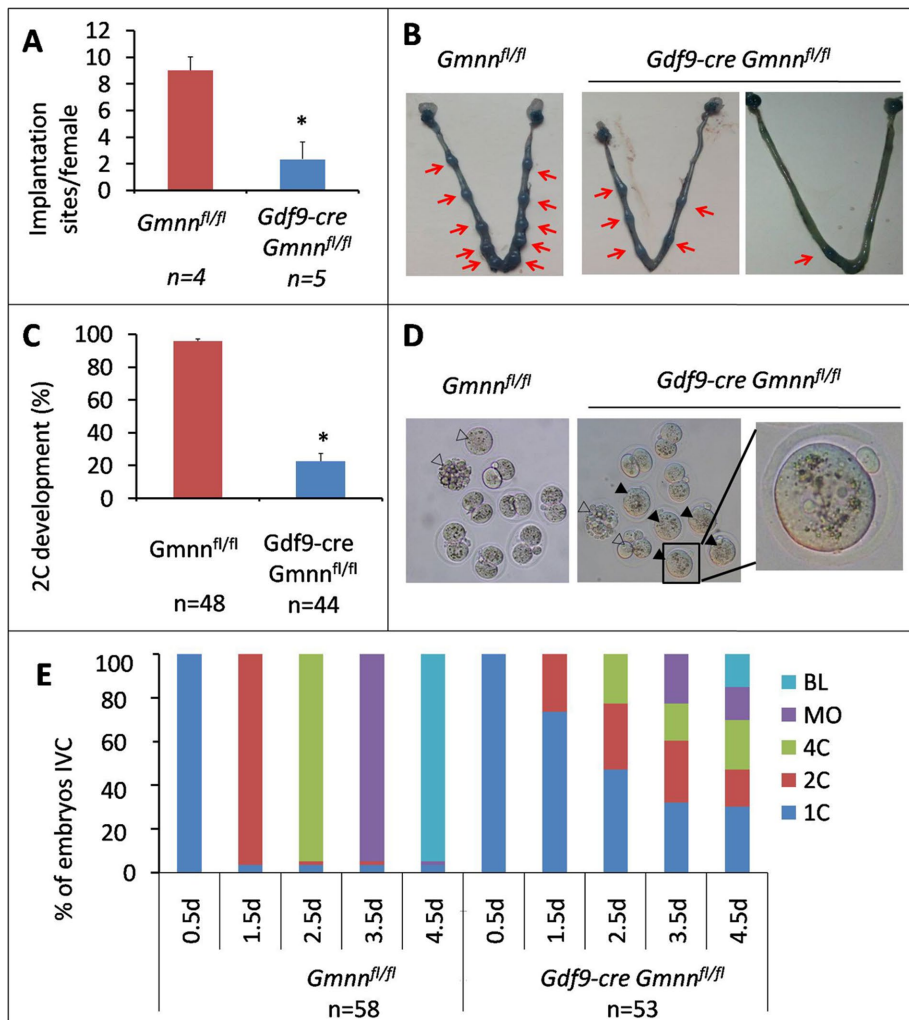
Previous research showed that depletion of geminin in *Xenopus* immature oocytes leads to a decrease of Cdt1 protein level during maturation and after activation of these oocytes (Narasimhachar and Coue, 2009). Geminin depletion induced rereplication and then prompted checkpoint activation in *Xenopus* (McGarry, 2002; Kerns et al., 2007, 2012) and U2OS cells (Klotz-Noack et al., 2012). The abnormal blastomeres with geminin depletion contain damaged DNA and undergo apoptosis (Hara et al., 2006). Therefore, in our study, we examined the protein level of Cdt1 and phosphorylated Chk1, Chk2, and H2AX in the mutant fertilized eggs by both immunofluorescence staining and Western blot analysis.

The immunofluorescence intensity of phosphorylated Chk1 in the mutant fertilized eggs was inconsistent (Figure 5B). Of 41 fertilized eggs from *Gdf9-cre Gmnn<sup>fl/fl</sup>* mice, 31 showed strong immunofluorescence intensity from *Gdf9-cre Gmnn<sup>fl/fl</sup>* mice, whereas only one of 42 eggs from *Gmnn<sup>fl/fl</sup>* mice showed strong staining (Figure 5C). Phosphorylated Chk2 displayed no signal,

and no difference between *Gdf9-cre Gmnn<sup>fl/fl</sup>* and the *Gmnn<sup>fl/fl</sup>* group was observed (Figure 5D). Most fertilized eggs from *Gdf9-cre Gmnn<sup>fl/fl</sup>* mice showed a stronger phosphorylated H2AX fluorescence signal than those from *Gmnn<sup>fl/fl</sup>* mice (Figure 5E). By Western blot detection, the fertilized eggs obtained from *Gdf9-cre Gmnn<sup>fl/fl</sup>* mice showed a decreased Cdt1 protein level but increased phosphorylated Chk1 and H2AX levels compared with the *Gmnn<sup>fl/fl</sup>* group (Figure 5F).

### DISCUSSION

The present study showed that oocyte-specific disruption of the geminin gene results in low fertility in mice. Although ovarian development, folliculogenesis, and ovulation of mutant mice are normal, the average pup number per litter, litter number, and total number of offspring in mutant mice are notably and dramatically decreased. Eggs can be fertilized, but most fertilized eggs show developmental delay, causing reduced embryo implantation. The changes of Cdt1



**FIGURE 4:** Early embryo development and implantation in *Gdf9-cre Gmnn<sup>fl/fl</sup>* female mice. (A) Average number of embryo implantation sites. Five female mice were examined after day 5 of pregnancy for each group. \* $p < 0.01$ . (B) Uterine imaging after intravenous injection of trypan blue, showing implantation sites. (C) Percentage of two-cell embryo development after insemination. \* $p < 0.01$ . (D) Bright-field images of in vivo two-cell embryos at 20 $\times$  magnification. There were seven two-cell embryos and two unfertilized eggs ( $\Delta$ ) in the *Gmnn<sup>fl/fl</sup>* group and three two-cell embryos, five fertilized eggs ( $\blacktriangle$ ), and two unfertilized eggs in the *Gdf9-cre Gmnn<sup>fl/fl</sup>* group. (E) Development status of naturally fertilized eggs after in vitro culture (IVC) for 4 d. Here 1C, 2C, 4C, MO, and BL indicate one-cell embryo, two-cell embryo, four-cell embryo, morula, and blastocyst, respectively.

protein and phosphorylated Chk1 and H2AX levels indicate that disruption of maternal geminin leads to DNA rereplication, which then activates the cell cycle checkpoint and DNA damage repair (Figure 6).

Previous studies showed that the main role of geminin is to bind and inhibit Cdt1 protein (Wohlschlegel et al., 2000; Benjamin et al., 2004; Lee et al., 2004; Saxena et al., 2004; Li and Blow, 2005; Lutzmann et al., 2006; Narasimhachar and Coue, 2009), which is responsible for strict one-time-only DNA replication during each S phase. The function of geminin is to maintain accurate DNA replication. Disruption of geminin protein may cause Cdt1 activation and DNA rereplication (Lin and Dutta, 2007; Rakotomalala et al., 2008). The abnormal DNA replication then activates the ATM/ATR signal pathway, which triggers the cell cycle checkpoint and results in increased phosphorylation of Chk1, as shown in our study (Figure 5, B and C). The inconsistent immunofluorescence intensity of phosphor-

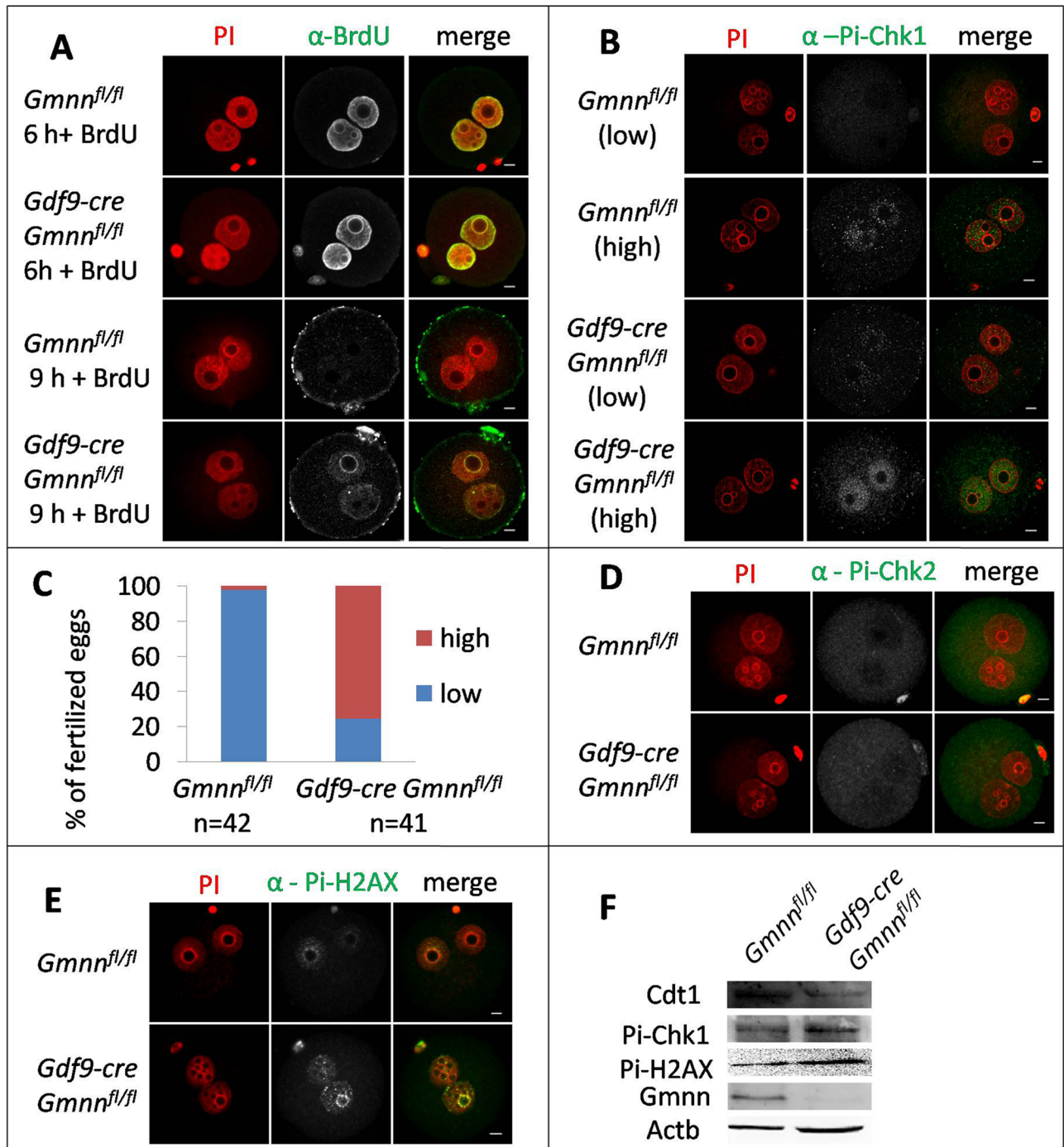
ylated Chk1 in the mutant fertilized eggs may be due to different levels of DNA rereplication caused by Cdt1 activation. Of interest, a limited number of fertilized eggs from *Gmnn<sup>fl/fl</sup>* mice to some degree also showed immunofluorescence staining of phosphorylated Chk1. The phosphorylated Chk1 in the control fertilized eggs might be induced by the accumulation of environmental DNA damage when oocytes experienced protracted arrest in meiotic prophase.

The phosphorylation level of Chk2 shows no increase (Figure 5D). The reason may be that the ATR/Chk1 pathway is activated at an early time point after the loss of geminin and DNA rereplication (Lin and Dutta, 2007). We infer that the abnormally repeated DNA replication produces a number of replication forks, which are accompanied by DNA breaks. The DNA breaks may recruit phosphorylated H2AX and switch on DNA damage repair. In addition, H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress (Ward and Chen, 2001) and DNA double-strand breaks (Burma et al., 2001), and DNA repair intermediates trigger H2AX phosphorylation via the ATR kinase (Hanasoge and Ljungman, 2007). The activated ATR/Chk1 pathway may also lead to the increase in H2AX phosphorylation, as indicated in our experiments.

Although the geminin protein is localized in the nucleus during meiosis and co-localizes with chromosomes in the MI stage, oocyte-specific knockout of the geminin gene does not affect the development and maturation of oocytes. In mitosis, geminin protein is localized to centrosomes and may play a role in the centrosome duplication process (Lu et al., 2009). The present study showed no localization of geminin protein at microtubule-organizing centers during the formation of spindles in both meiosis I and meiosis II. Of note, there is no centriole duplication

in the process of oocyte meiotic maturation. Therefore geminin has no obvious function in the process of oocyte meiotic maturation. In addition, our results indicate that geminin deletion in mouse oocytes does not disrupt oocyte development, meiotic maturation, ovulation, or subsequent fertilization. Previous research found that Cdc6 is the only missing replication factor whose translation is necessary and sufficient to confer DNA replication competence to the egg before fertilization (Lemaitre et al., 2002; Anger et al., 2005). Therefore geminin depletion and Cdt1 activation still could not cause DNA rereplication in mouse oocytes. That would be the reason for the absence of significant effect of geminin depletion during oogenesis and oocyte meiotic maturation.

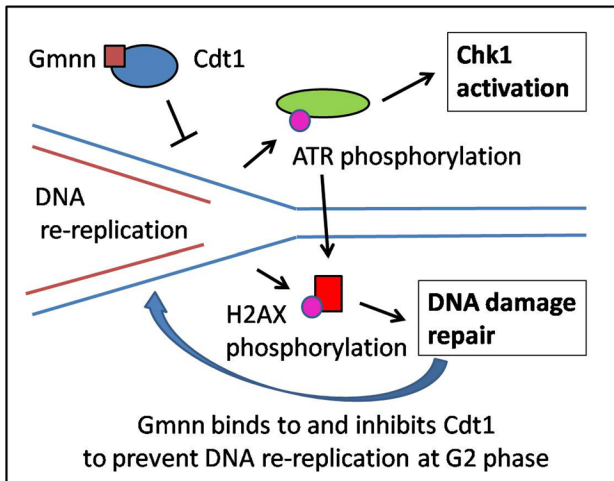
The mutant mice display significantly decreased reproduction, but they are still fertile. Two reasons for the fertility are surmised: one possibility is the incomplete knockout of the geminin gene, and



**FIGURE 5:** Geminin depletion causes DNA rereplication, decreased Cdt1 expression and increased Chk1, and H2AX phosphorylation in mutant fertilized eggs. (A) Confocal micrographs showing BrdU labeling (green) in the fertilized eggs at 14 h of IVF. Here, 6 h + BrdU and 9 h + BrdU indicate fertilized eggs that were moved into HTF medium supplemented with BrdU at 6 and 9 h of IVF, respectively. (B) Immunostaining for the phosphorylated Chk1 in mutant fertilized eggs at late zygote stage. DNA was counterstained with PI. Bar, 10  $\mu$ m. (C) Percentage of fertilized eggs with different immunofluorescence intensities of phosphorylated Chk1 (low or high as shown in B). (D) Immunostaining of phosphorylated Chk2 in mutant fertilized eggs at late zygote stage. (E) Immunostaining for the phosphorylated H2AX in mutant fertilized eggs at late zygote stage. (F) The protein levels in the mutant fertilized eggs detected by Western blot.

the second is the existence of compensatory mechanisms after geminin gene knockout. By genotyping, it was found that all of the offspring of the mutant females generate the deletion of the floxed geminin allele (unpublished data). These results indicate that the

knockout efficiency of *Gdf9-Cre* transgenic mice is very high. Another reason may be that low-level DNA damage induced by geminin depletion can be rescued by the DNA repair mechanism. The inconsistent immunofluorescence intensity of phosphorylated



**FIGURE 6:** Schematic showing the possible mechanism of geminin function in fertilized eggs.

Chk1 (as shown in Figure 5, B and C) may indicate the different development potency of the mutant fertilized eggs. Once the embryos overcome the injury of maternal geminin depletion, they can develop to normal individuals.

In summary, maternal geminin does not regulate oogenesis and oocyte meiotic maturation, but it does control accurate DNA replication and timely cleavage of fertilized eggs.

## MATERIALS AND METHODS

### Mice

Geminin flox/flox (*Gmnn<sup>flox</sup>*) mice (Shinnick *et al.*, 2010) were maintained with a mixed genomic background of 129S4/SvJae and C57BL/6J (016913; Jackson Laboratory, Bar Harbor, ME), and *Gdf9-cre* mice (Lan *et al.*, 2004) were maintained with C57/BL6 genomic background. Mutant mice were homozygous for the geminin floxed allele and heterozygous for *Gdf9-cre* (*Gdf9-cre Gmnn<sup>flox</sup>*), and control mice were homozygous for Geminin floxed allele and *Gdf9-cre* negative (*Gmnn<sup>flox</sup>*). Mice were housed in 12-h alternating light/dark cycles, with free access to water and food.

All experiments were conducted with the approval of the Animal Research Committee of the Institute of Zoology, Chinese Academy of Sciences. Mice were killed under standard protocols, and all efforts were made to minimize suffering.

### Oocyte collection and culture

The fully-grown GV-stage oocytes (>80  $\mu\text{m}$ ) were isolated from ovaries of 6- to 9-wk-old female mice and cultured in M2 medium (Sigma-Aldrich, St. Louis, MO) under paraffin oil at 37°C and 5% CO<sub>2</sub> in air. They were collected at different times of culture for immunofluorescence staining. The selected times were 0 h (GV), 4 h (GVBD), 8 h (MI), and 12 h (MII).

### Fertility and natural ovulation analysis

To evaluate the reproductive activity, seven individually housed *Gmnn<sup>flox</sup>* and *Gdf9-cre Gmnn<sup>flox</sup>* female mice at the age of 6 wk were crossed to *Gmnn<sup>flox</sup>* male mice with known fertility. The numbers of pups and litters were recorded up to 6 mo. For natural ovulation, female mice in estrus were mated with *Gmnn<sup>flox</sup>* or wild male mice. The next morning, female mice with plugs were killed, and fertilized eggs were separated from the oviduct and counted. The fertilized eggs were cultured in GM medium (LifeGlobal) for further develop-

ment. The in vivo two-cell embryos were separated and counted at day 2 after mating.

For IVF, spermatozoa were collected from the adult *Gmnn<sup>flox</sup>* or wild males and preincubated in HTF medium for 1 h in an atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C. Superovulated MII oocytes were collected and inseminated with capacitated spermatozoa. Two hours after insemination, fertilized eggs were washed and collected at the times indicated.

### Embryo implantation site detection

Female mice were mated with fertile males to induce pregnancy. After day 5 of pregnancy, implantation sites were visualized by intravenous injections of trypan blue. The uterine tissue was separated and contained, and the implantation sites were counted.

### Histological analysis of ovaries

Ovaries used for histological analysis were fixed in 4% paraformaldehyde (pH 7.5) overnight at 4°C, dehydrated in a graded ethanol series, cleared in xylene, and embedded in paraffin wax. The paraffin-embedded ovaries were sectioned serially at 8  $\mu\text{m}$  for hematoxylin and eosin staining.

### Quantitative real time-PCR

Approximately 60 oocytes or embryos for each group were used to extract RNA for reverse transcription reactions. Expression level of geminin was validated by quantitative real-time PCR analysis (Roche 480) according to the manufacturer's instructions. Primers for geminin were the 5'-ACGGATGCTAGGCCGTGTAC-3' (forward) and 5'-GCA CCGTGTAGTTAGTTTACCAAGAG-3' (reverse). The reference gene was  $\beta$ -actin (*actb*), and primers were 5'-GGCTGTATCCCCTC-CATCG-3' (forward) and 5'-CCAGTTGGTAACA ATGCC ATGT-3' (reverse). The experiments were repeated at least three times.

### Western blot analysis

A total of 200 mouse oocytes or zygotes per sample were mixed with SDS sample buffer and boiled for 5 min at 100°C for SDS-PAGE. Western blotting was performed as described previously (Zhang *et al.*, 2004) using the antibody dilution anti-geminin (BS7535; Bioworld, St. Louis Park, MN) at 1:100; anti-actb (TA-09; Zhongshan Golden Bridge Biotechnology, Beijing, China) at 1:1000; anti-Cdt1 (07-1383; Millipore, Darmstadt, Germany) at 1:1000; anti-Pi-Chk1S345 (2348; Cell Signaling Technology, Danvers, MA) at 1:1000; and anti-Pi-H2AXS139 (9718; Cell Signaling Technology) at 1:1000. The membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000; ZB2301 and ZB2305; Zhongshan Golden Bridge Biotechnology) for 1 h at 37°C. Protein bands were detected using Thermo Supersignal West Pico chemiluminescent substrate.

### BrdU labeling for DNA replication

Fertilized oocytes were labeled in vitro with BrdU in order to confirm DNA replication. The zygotes were incubated in HTF medium supplemented with 100  $\mu\text{M}$  BrdU (858811; Sigma-Aldrich) in an atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C. After incubation, the zygotes were fixed for 15 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.5% Triton X-100 in PBS for 20 min at room temperature, treated with 4 N HCl at room temperature for 10 min, and subsequently neutralized for 10 min with 100 mM Tris-HCl buffer (pH 8.5) after permeabilization. After blocking at 4°C overnight with 0.2% Tween 20 in PBS/bovine serum albumin (BSA; PBS containing 10 mg/ml BSA), the cells were incubated for 1 h at room temperature with anti-BrdU

antibody (1:1000;B8434; Sigma-Aldrich), followed by secondary Alexa Fluor 488–conjugated antibody (1:1000; A11001; Life Technologies, Shanghai, China).

### Immunofluorescence and confocal microscopy

Oocytes and embryos were fixed for 1 h in 3.7% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS for 20 min at room temperature. Then the cells were incubated at 4°C overnight with primary antibodies (geminin at 1:50 [sc8449; Santa Cruz Biotechnology, Dallas, TX], Pi-H2AXS139 [9718] and Pi-Chk1S345 [2348] at 1:200 [Cell Signaling Technology], and Pi-Chk2T68 at 1:200 [BS4043; Bioworld]) and then incubated for 1 h with a secondary Alexa Fluor 488–conjugated antibody (1:1000; A11008 and A11055; Life Technologies) or Alexa Fluor 594–conjugated antibody (1:1000; A11058; Life Technologies). For  $\alpha$ -tubulin staining, the oocytes were incubated with only the anti- $\alpha$ -tubulin–fluorescein isothiocyanate antibody (1:1000; F2168; Sigma-Aldrich) for 2 h at room temperature. DNA was stained for 20 min with 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI). Fluorescence was detected using a Zeiss LSM710 laser-scanning confocal microscope.

### Data analysis

Statistical analysis was performed using SPSS (SPSS China). Data were expressed as mean  $\pm$  SEM, and  $p < 0.05$  was considered as statistically significant. All experiments were repeated at least three times.

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