Preimplantation Mouse Embryos Depend on Inhibitory Phosphorylation of Separase To Prevent Chromosome Misregulation

Xingxu Huang,1* Claudia V. Andreu-Vieyra,2 Meizhi Wang,1 Austin J. Cooney,3 Martin M. Matzuk,4 and Pumin Zhang4,5*

Model Animal Research Center, Nanjing University, Nanjing, China,1 and Department of Pathology,2 Department of Cell and Molecular Biology,3 Department of Molecular Physiology and Biophysics,4 and Department of Biochemistry and Molecular Biology,5 Baylor College of Medicine, Houston, Texas 77030

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Separase is a critical protease that catalyzes the cleavage of sister chromatid cohesins to allow the separation of sister chromatids in the anaphase. Its activity must be inhibited prior to the onset of the anaphase. Two inhibitory mechanisms exist in vertebrates that block the protease activity. One mechanism is through binding and inhibition by securin, and another is phosphorylation on Ser1126 (in humans [Ser1121 in mice]). These two mechanisms are largely redundant. However, phosphorylation on Ser1121 is critical for the prevention of premature sister separation in embryonic germ cells. As a result, Ser1121-to-Ala mutation leads to deletion of germ cells in development and subsequently to infertility in mice. Here, we report that the same mutation also causes embryogenesis failure between the 8- and 16-cell stages in mice. Our results indicate a critical role of separase phosphorylation in germ cell development as well as in early embryogenesis. Thus, deregulation of separase may be a significant contributor to infertility in humans.

Sister chromatids are held together by a multisubunit complex called cohesin composed of Smc1 and -3 and Scc1 and -3 (24). To separate the sister chromosomes, cohesin complexes are removed in a two-step process. First, cohesins on chromosome arms are removed by Plk1- and Aurora B-mediated phosphorylation before the anaphase (4, 8, 19, 20, 31, 35). Second, the centromere-localized cohesins, which are protected by Sgo and PP2A from phosphorylation-mediated removal (13, 21, 28, 29, 32), are cleaved by a protease called separase at the onset of the anaphase (33, 34). Prior to the anaphase, separase is inhibited by securin and by phosphorylation, which is most likely catalyzed by cyclin B1/Cdk1. Phosphorylation by cyclin B1/Cdk1 per se is not inhibitory to separase. Rather, the phosphorylation allows the binding of cyclin B1/Cdk1 as an inhibitor to separase (5). Two phosphorylation sites in separase, Ser1126 and Thr1326 (Ser1121 and Thr1321 in mice, respectively), that are important for the inhibition have been identified (30). Activation of separase depends on the function of the E3 ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C), since both securin and cyclin B1 are substrates of APC/C (1–3, 15, 16, 23, 25, 27, 36). Given that APC/C is inhibited by the spindle assembly checkpoint, separation of sister chromatids therefore cannot occur until the checkpoint is satisfied. Thus, the spindle assembly checkpoint prevents premature sister separation and ensures chromosomal stability.

Misregulation of chromosomes has dire consequences. It causes genetic imbalances that may transform cells and lead to cancer development in somatic tissues. In germ lines, misregulation in either meiosis I, mainly manifested as nondisjunction of homologous chromosomes, or meiosis II, manifested as premature sister chromatid separation, will generate aneuploid gametes, directly affecting the fecundity of an organism (26, 37). Although the molecular mechanisms underlying chromosome segregation errors in meiosis are still not clear (6), deregulation of separase, either directly or indirectly, is likely a significant contributor.

We previously showed that securin and separase phosphorylation are redundant in almost all somatic tissues, as mice lacking either separase inhibitory mechanism are essentially normal (9, 22). However, phosphorylation of separase is uniquely required during germ line development (9). Mice carrying a nonphosphorylatable separase (S1121A) allele are sterile, largely due to depletion of germ cells during embryogenesis. The failure of the germ cells to reach sexually mature stages in the mutant mice prevented us from assessing the function of the inhibitory phosphorylation of separase in meiosis. Here we report our analysis of mice with an oocyte-specific S1121A mutation in separase. We found that these mice were still infertile. However, the infertility was not a result of meiotic errors caused by the mutant separase but was rather a failure of early embryogenesis of zygotes carrying the mutant allele prior to the 16-cell stage.

MATERIALS AND METHODS

Generation and analysis of oocyte-specific S1121A-separase mice. The conditional separaseS1121A allele was described previously. To create oocyte-specific
S1121A-separate mice, separate+/S1121A-flox-puro mice were crossed with Zp3-Cre transgenic mice (17).

Standard histological procedures were followed to prepare ovaries. In brief, ovaries were fixed in 10% neutral buffered formalin (Sigma). The specimens were dehydrated through a graded series of ethanol washes, cleared in Histoclear, embedded in paraffin, and sectioned. Sections (4 μm thick) were dewaxed and stained with periodic acid-Schiff–hematoxylin or hematoxylin and eosin.

Antibodies and immunological analyses. Primary antibodies used for immunofluorescence and Western blotting were mouse anti-α-tubulin (Sigma), mouse anti-β-tubulin (clone E7; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), mouse anti-cyclin B1, rabbit anti-active caspase-3, and mouse antiserine (Novocastra, Newcastle, United Kingdom). The following secondary antibodies were used: Cy3- and fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G and Cy3- and fluorescein isothiocyanate-conjugated anti-mouse antibodies (Jackson ImmunoResearch Laboratories, Inc.).

For Western blotting, equal amounts of protein extracts from embryos were separated for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The blots were probed with the indicated primary and appropriate secondary antibodies (Bio-Rad). Immunocomplexes were detected with ECL chemiluminescence (GE Healthcare, Piscataway, NJ).

Collection and culture of oocytes and preimplantation embryos. For meiosis I (MI) oocytes, ovaries were isolated from 4- to 6-week-old female mice after 46 to 48 h of treatment with 5 IU of pregnant mare serum gonadotropin (Calbiochem). Cumulus-free, fully grown, granulosa cell-intact oocytes were released by puncturing antral follicles with a fine needle in M2 medium (Sigma). For MI oocytes, 4- to 6-week-old female mice were superovulated by intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin followed by intraperitoneal injection of 5 IU of human chorionic gonadotropin (Calbiochem) 46 h later. MII oocytes were flushed out from the oviducts in M2 medium and then cultured in M16 medium (Sigma) in a 5% CO2 humidified incubator at 37°C. To arrest the cells in prometaphase, 5 μM nocodazole was added to the culture medium for 3 h.

To collect early embryos, female mice were superovulated as described above and mated immediately with stud male mice. Embryos were flushed out from the oviducts or uteri in M2 medium and cultured at 37°C in minimal essential medium (Invitrogen) containing 20% fetal calf serum in a humidified incubator.

RESULTS

Inhibitory phosphorylation of separase is not required for meiosis. We recently reported that both male and female separase+/S1121A mice are sterile (9). Because of the sterility, the separase+/S1121A allele could not be transmitted through the germ line. We could obtain the mutants for study only by crossing separate+/S1121A-flox-puro mice with a Cre deleter strain of mice. We have used Meox2+/Cre mice to produce doubly heterozygous mice (Meox2+/S1121A-flox-puro) in which separate+/S1121A-flox-puro was converted to separate+/S1121A through the action of Cre recombinase in most of the cells (9). In males, primordial germ cells were lost to premature sister chromatid separation and subsequent apoptosis between embryonic days 11.5 (E11.5) and E14.5. This result indicates that embryonic germ cells rely primarily on phosphorylation to inhibit separase, which is in agreement with the observation that these cells express relatively low levels of securin, the inhibitor of separase (9). In females, although there were the losses of primordial germ cells seen with males, a substantial number of primordial germ cells did survive. At present, it is not clear whether this differential response in females to the loss of inhibitory phosphorylation of separase is caused by sexual dimorphism.

Having established that the inhibitory phosphorylation of separase plays an essential role in the mitoses of embryonic germ cells, we asked whether there is a requirement for this inhibitory mechanism on separase in meiosis. To test that, we activated the S1121A mutant allele of separase in oocytes by crossing separate+/S1121A-flox-puro mice with Zp3-Cre transgenic mice in which Cre activity is expressed in oocytes 5 days after birth (17). At this stage, the oocytes are arrested in the prophase of meiosis I and will resume meiosis after sex maturation.

To determine whether S1121A-separate has an impact on ovary development, we first examined this female reproductive organ histologically in separate+/+ and separate+/S1121A-flox-puro Zp3-Cre (hereafter referred as control) and separate+/S1121A-flox-puro Zp3-Cre (hereafter referred as mutant) mice. At 3 weeks and 6 months of age, there was no apparent difference between control and mutant results (Fig. 1A). The control and mutant organs contained similar numbers of follicles and produced similar numbers of oocytes upon superovulation (Fig. 1B). PCR genotyping of the oocytes demonstrated the presence of separate S1121A allele in the mutant (Fig. 1C).

If the loss of the inhibitory phosphorylation of separase...
causes problems in meiotic divisions, for example, premature homologue separation in metaphase I or premature sister chromatid separation in metaphase II, as it did in mitosis of embryonic germ cells (9), it is expected then that separase\(^+\)/S1121A mice would be sterile. Although the mutants seemed able to produce oocytes carrying a separase\(^+/\)S1121A allele (Fig. 1C), these oocytes might have suffered meiotic errors and might not be able to be fertilized or to develop upon fertilization. We therefore subjected female separase\(^+\)/S1121A-flox-puro Zp3-Cre mice to the fecundity study. At 6 weeks of age, these mice were housed together with stud males. The progeny from the mating were genotyped and recorded. Out of 82 pups in 12 litters, 73 were separase\(^+/\)+ and 9 were separase\(^+\)/S1121A, but none were separase\(^+/\)/S1121A.

These breeding data demonstrate that the mutant allele could not be transmitted to the next generation. The small number of separase\(^+/\)/S1121A mice die during embryonic development. We performed in vitro embryo culture experiments to determine more precisely when the lethality of separase\(^+/\)/S1121A embryos occurred. Two-cell embryos (E1.5) were collected from control and mutant females and cultured for up to 48 h. At 12, 36, and 48 h, we counted the number of live embryos obtained from 4-week-old superovulated females. Since separase\(^+/\)+ would have been produced. The former could proceed through the rest of the development, be fertilized by separase\(^+\)+ sperms, and produce separase\(^+/\)+ offspring. Since we did not obtain any offspring carrying a separase\(^+/\)/S1121A allele, separase\(^+/\)/S1121A oocytes could not complete meiosis II normally or could not be fertilized, or the zygote (separase\(^+/\)/S1121A) failed to develop. We tested each of these three possibilities.

We cultured in vitro the oocytes isolated from both control and mutant mice. Polar body extrusions were quantified, and no differences in the results were detected (Fig. 2A). Furthermore, normal MI bivalent and MII univalent chromosome configurations were observed (Fig. 2B) and we could not detect any increases in the premature separation of either homologue chromosomes or sister chromatids in the mutant oocyte results compared to the control results. These data suggested that separase\(^+/\)/S1121A oocytes completed MI normally and that after MI, both separase\(^+\)+ and separase\(^+/\)/S1121A oocytes could also complete MII normally. In other words, S1121A-separase did not disturb meiosis. Therefore, we tested the ability of the mutant oocytes to be fertilized. We harvested two-cell embryos from superovulated control and mutant females mated with wild-type males. Similar numbers of embryos per female were obtained (Fig. 2D), indicating that separase\(^+/\)/S1121A oocytes could develop into functional gametes and be fertilized. Otherwise, we would see a reduction by half of the numbers of embryos produced (because separase\(^+\)+ oocytes would still be able to produce embryos in the mutant females). Taken together, these results demonstrate that the inhibitory phosphorylation is required neither for meiosis nor for fertilization, at least not when the mutant allele is present in a heterozygous state. This left us with only the possibility that separase\(^+/\)/S1121A zygotes failed to develop to account for the fact that no such animals were born.

**Early embryogenesis failure in separase\(^+/\)/S1121A mice.** To determine when separase\(^+/\)/S1121A mice die during embryonic development, we performed timed mating analysis. The mutant (separase\(^+/\)/S1121A-flox-puro ZF3-Cre) females were mated with wild-type males, and the resulting embryos were collected at different stages. Based on the Mendelian laws of inheritance, half of the progeny from the mating would be wild type (separase\(^+/\)+) and half would be heterozygous (separase\(^+/\)/S1121A). In examinations of over 120 E3.5-to-E7.5 embryos, we could not detect the mutant allele, suggesting that separase\(^+/\)/S1121A embryos died prior to the blastocyst (E3.5) stage. Microscopic observation indicated that by E3.5 there were many dead or dying embryos in the mutants (Fig. 3A and B).

Next, we performed in vitro embryo culture experiments to determine more precisely when the lethality of separase\(^+/\)/S1121A embryos occurred. Two-cell embryos (E1.5) were collected from control and mutant females and cultured for up to 48 h. At 12, 36, and 48 h, we counted the number of live embryos. FIG. 2. Inhibitory phosphorylation of separase is not required for meiosis. (A) Analysis of polar body (PB) extrusions in in vitro cultured oocytes. (B) Chromosome spreads of MI and MII oocytes from 4-week-old mice. (C) Quantitative analysis of two-cell embryos produced from 4-week-old superovulated females.
embryos under a microscope. As shown in Fig. 3C, after 12 h in culture, more than 80% of the embryos divided and arrived at the four-cell stage, although a minor fraction of embryos died. There were no differences between control and mutant results in either the numbers of four-cell embryos or the numbers of embryos that died. By 36 h, however, there were significantly more embryos that had arrived at the 16-cell stage in the control group compared to the mutant group results. Importantly, at this time point, there were more embryos lagging behind development in the mutant than in the control group (Fig. 3C). The percentages of mutant embryos that were still 4 cells, between 4 and 8 cells, or between 8 and 16 cells in size were significantly higher than those of control embryos. Those that were lagging behind contained dead/fragmented cells (Fig. 3D). As a result, more dead embryos were found in the mutant than in the control group. Similar situations were observed after 48 h of culture, when significantly fewer embryos reached the blastocyst stage in the mutant than in the control group (Fig. 3C). Those that did reach the blastocyst stage in the mutant group were presumably the separase+/+ embryos. These data indicate that separase+/S1121A embryos died between the 8- and 16-cell stages. Indeed, genotyping of pooled live embryos at different stages demonstrated that the separaseS1121A allele was absent by the 16-cell stage (Fig. 3E).

**Aberrant mitosis in early separase+/S1121A embryos.** Given that S1121A-separase causes precocious sister chromatid separation and associated mitotic problems in embryonic germ...
cells, we wondered whether it was causing similar problems in early embryos. To that end, we immunostained eight-cell embryos obtained from in vitro culturing. Indeed, abnormal mitotic configurations, including misaligned chromosomes in the metaphase and lagging chromosomes in the anaphase (Fig. 4A), were frequently observed in the mutant group and the frequency of abnormal mitoses was much higher in the mutant than in the control group (Fig. 4B). These results suggested that the mutant separase caused premature sister separation.

To confirm that, we cultured eight-cell embryos for 3 h in the presence of nocodazole and spread chromosomes. As shown in Fig. 4C, sister chromatids stayed together in the control group but separated in the mutant group.

We not only observed abnormal mitoses in the mutant group but also observed a large number of embryos containing cells with micronuclei in the mutant group (Fig. 5A and B). The micronuclei were most likely formed by prematurely separated and missegregated chromosomes. As a result, these cells were highly abnormal and very likely to be aneuploid and were targets of apoptotic cell death (38). Indeed, as in the embryonic germ cell results, S1121A-separase caused increased apoptosis in early embryos, as detected by active caspase-3 staining (Fig. 5C and D).

Low levels of securin expression in early embryos necessitate the requirement of inhibitory phosphorylation of separase. The essentiality of the inhibitory phosphorylation of separase in early embryogenesis conflicted with the redundancy provided by securin. Perhaps the cells in early embryos express low levels of securin as embryonic germ cells so that they rely on the phosphorylation to inhibit separase. To test that possibility, we collected 200 four-cell and 100 eight-cell wild-type embryos and measured the level of securin protein by Western blot analysis. In agreement with the observation that the mutant embryos died between the 8- and 16-cell stages, we
found that 8-cell embryos contained about half of the amount of securin that the 4-cell embryos contained (Fig. 6A and B). Further, immunostaining demonstrated that securin levels were highest in two-cell embryos and declined with time (Fig. 6C and D). These data suggest that the redundancy between securin and phosphorylation of separase is lost in early embryos as seen in embryonic germ cells due to reduced levels of securin expression.

**DISCUSSION**

Genome stability is essential to an organism. Chromosome missegregation leads to aneuploidy, a form of genome instability (7, 11, 14, 23). Elaborated mechanisms have evolved to ensure faithful segregation of genetic materials. In eukaryotes, a key mechanism that prevents chromosome missegregation is that of the spindle assembly checkpoint, which blocks sister chromatid separation before all chromosomes are aligned at metaphase plate and are properly attached by the spindle microtubules. The separation of sister chromatids requires the protease activity of separase to destroy sister cohesion in the centromere region. The spindle assembly checkpoint inhibits APC/C, the E3 ubiquitin ligase that relieves inhibition on separase, thereby restricting the resolution of sister cohesion until the checkpoint is satisfied.

In contrast to budding yeast separase, vertebrate separase is inhibited by two mechanisms, securin binding and phosphorylation (30). These two mechanisms are largely redundant (9, 22). However, the redundancy is not ubiquitous, as we showed previously that embryonic germ cells rely on the phosphorylation of separase to prevent premature separation of sister chromatids and here that the phosphorylation is similarly required in early embryos. In both cases, the apparent low levels of securin expression seemed to underlie the requirement for separase phosphorylation. We do not know the reason why securin levels decline as the zygote develops (Fig. 6). It is possible that zygotic expression of securin is not turned on until some point beyond the 16-cell stage. Before that point, securin expression might depend on the maternal supply. So the levels are highest at the two-cell stage and decrease as the supply is diluted/exhausted with each cell division. The fact that Meox2+/Cre; Separase+/S1121A-flox-puro mice are viable indicates that after E5.5 (when Meox2-Cre is turned on in most lineages), the phosphorylation of separase is no longer essential, most likely because of the redundancy provided by securin (9).
The finding that separase<sup>S1121A</sup> causes lethality in embryonic germ cells and in cells of preimplantation embryos is in sharp contrast with the fact that securin<sup>/H11002</sup>/separase<sup>/H11001</sup>/S1121Amouse embryonic stem (ES) cells are essentially normal (10). Separase<sup>S1121A</sup> did not cause lethality in ES cells even when securin was completely deleted, suggesting that there were other mechanisms (perhaps unique to ES cells) that either inhibit separase or protect sister chromatid cohesion. However, these cells were sensitive to nocodazole. They failed to recover from nocodazole treatment due to precocious sister chromatid separation. Thus, the other mechanisms might be sufficient to maintain the viability of securin<sup>-/-</sup>/separase<sup>-/-/S1121A</sup> ES cells but were unable to maintain sister chromatid cohesion under conditions requiring that the sisters remain unseparated over extended periods of time. The nature of these other mechanisms in ES cells is a subject for future investigation.

A leading genetic cause of human fertility failure is aneuploidy. Approximately 10 to 30% of human zygotes and 50% of spontaneous abortuses have an abnormal number of chromosomes (12). Aneuploidy can result from errors in meiotic chromosome segregation during gametogenesis or errors in postzygotic mitotic chromosome segregation during early development (7). Our studies revealed the critical role of separase phosphorylation in germ cell development and in early embryogenesis. Failure to control separase results in infertility in mice. It is possible that loss of separase control contributes to infertility in humans.

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