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RESEARCH ARTICLE

Cyclic AMP in oocytes controls meiotic prophase I and primordial folliculogenesis in the perinatal mouse ovary

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ABSTRACT

In mammalian ovaries, a fixed population of primordial follicles forms during the perinatal stage and the oocytes contained within are arrested at the dictyate stage of meiotic prophase I. In the current study, we provide evidence that the level of cyclic AMP (cAMP) in oocytes regulates oocyte meiotic prophase I and primordial folliculogenesis in the perinatal mouse ovary. Our results show that the early meiotic development of oocytes is closely correlated with increased levels of intra-oocyte cAMP. Inhibiting cAMP synthesis in fetal ovaries delayed oocyte meiotic progression and inhibited the disassembly and degradation of synaptonemal complex protein 1. In addition, inhibiting cAMP synthesis in *in vitro* cultured fetal ovaries prevented primordial follicle formation. Finally, using an *in situ* oocyte chromosome analysis approach, we found that the dictyate arrest of oocytes is essential for primordial follicle formation under physiological conditions. Taken together, these results suggest a role for cAMP in early meiotic development and primordial follicle formation in the mouse ovary.

KEY WORDS: cAMP, Meiotic prophase I, Oocytes, Primordial follicle formation

INTRODUCTION

In mammals, the oocytes that are generated early in life represent the entirety of female reproductive potential over the life span (Faddy et al., 1992; Kezele et al., 2002). To maintain an immature oocyte in a dormant state, it is enclosed by several flattened pregranulosa cells to establish a functional unit called the primordial follicle, which forms in the fetal or neonatal ovary (Pepling, 2012). Proper oogenesis and folliculogenesis in the perinatal ovary are essential for fertility; however, the mechanisms regulating these processes remain unclear.

In mice, primordial germ cells (PGCs), which are oocyte progenitors, migrate to the genital ridge and form the germline cyst by rapidly dividing during embryonic development (Ginsburg et al., 1990; Edson et al., 2009; Pepling, 2012). Retinoic acid then stimulates the PGCs in the ovary to enter meiosis at ∼13.5 days post coitum (dpc) (Bowles et al., 2006; Bowles and Koopman, 2007). The female germ cells are then referred to as oocytes (Pepling, 2006). The oocytes then progress through the leptotene, zygotene,

pachytene and diplotene stages of meiotic prophase I, and arrest at the dictyate stage in the neonatal ovary (Slizynski, 1957; Borum, 1961). It is crucial for fertility that oocytes undergo the correct meiotic progression in the perinatal ovary. Forcing oocytes to undergo abnormal meiosis by deleting meiosis-related genes, such as *Dazl*, *Spo11*, *Dmc1*, *Atm*, *Msh4* and *Msh5*, leads to infertility in mice (Pepling, 2006; Edson et al., 2009). However, the upstream signaling mechanism that regulates early oocyte meiosis in the perinatal ovary remains unknown.

Cyclic AMP (cAMP) is a well-characterized intracellular second messenger that is involved in many biological processes, including oogenesis. In mammals, the concentration of intra-oocyte cAMP plays a pivotal role in controlling the arrest and resumption of meiosis in oocytes in the adult ovary (Mehlmann et al., 2004; Zhang and Xia, 2012). A high concentration of cAMP produced by both oocytes and cumulus cells maintains the meiotic arrest of immature oocytes, whereas a decrease in cAMP concentration in oocytes leads to the resumption of meiosis (Conti et al., 2012). Although the function of cAMP in regulating late stages of meiosis in activated oocytes has been studied extensively, it is not known whether cAMP contributes to early oocyte meiosis and what the mechanism of its involvement might be.

Recently, a series of studies identified several cellular factors and pathways that are crucial for regulating the formation of primordial follicles (Pepling, 2012). For example, the Notch pathway has been shown to regulate the breakdown of germline cysts and the assembly of primordial follicles in mice (Trombly et al., 2009; Guo et al., 2012; Manosalva et al., 2013; Vanorny et al., 2014), and overactive KIT signaling has been shown to accelerate cyst breakdown in cultured fetal mouse ovaries (Jones and Pepling, 2013). Progesterone (P4) and estradiol (E2) have also been shown to have roles in controlling the formation of primordial follicles in several mammalian species (Chen et al., 2007; Dutta et al., 2014). A number of additional growth factors, such as nerve growth factor (NGF) (Dissen et al., 2001; Abir et al., 2005; Chaves et al., 2013) and connective tissue growth factor (CTGF) (Schindler et al., 2010), have also been reported to participate in primordial follicle formation and development (Pepling, 2012). Less is known about the relationship between early meiosis and primordial follicle formation. The premature loss of synaptonemal complex protein 1 (SYCP1) has been reported to increase the number of oocytes entering the diplotene stage and accelerate primordial follicle formation in rodents (Paredes et al., 2005), suggesting that the progress of oocyte meiosis is correlated with the formation of primordial follicles. However, the relationship between oocyte meiosis and primordial follicle formation under physiological conditions is not well understood.

In this study, we investigated the function of cAMP in regulating early meiosis and the formation of primordial follicles in the perinatal mouse ovary. We show that cAMP produced by the oocyte

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DEVELOPMENT

controls the meiotic process in the fetal ovary, and that cAMP potentially regulates the disassembly and degradation of SYCP1 in oocytes. By regulating oocyte meiosis, cAMP actively participates in establishing the primordial follicle pool. Moreover, we show that meiotic arrest of oocytes is crucial for primordial follicle formation in the mouse ovary under physiological conditions.

RESULTS
The level of cAMP in oocytes correlates with meiotic prophase I in the mouse perinatal ovary

To determine whether cAMP contributes to the regulation of early oocyte meiosis and folliculogenesis, we measured the level of cAMP in perinatal ovaries from 15.5 dpc to 4 days post partum (dpp) by radioimmunoassay. Significant changes were observed in cAMP levels during ovarian development at the perinatal stage (Fig. 1A). The level of cAMP markedly increased from 15.5 dpc to 17.5 dpc. After labor, ovarian cAMP remained at a high level at 1 dpp and 4 dpp. These results suggest that the level of cAMP increases to high levels in the ovary throughout early oogenesis.

To investigate the potential relationship between the increasing level of ovarian cAMP and the early meiotic development of oocytes, we next assessed the relationship between cAMP levels and the progression of oocyte meiosis in perinatal mouse ovaries using chromosome spreads and synaptonemal complex protein 3

(SYCP3) staining. The stages of prophase I were defined by the appearance of axial elements as previously described (Fig. 1B) (Beaumont and Mandl, 1962; Peters et al., 1997; Prieto et al., 2004). The dictyate stage was identified by the presence of two to four clearly visible nucleoli and chromosomes that were decondensed and diffuse (Bakken and McClanahan, 1978; Hartung et al., 1979). At 15.5 dpc, the majority of oocytes were in the zygotene stage of meiosis (Fig. 1C). Concurrent with the increase in cAMP at 17.5 dpc (Fig. 1A), the oocytes were predominantly at the pachytene stage (Fig. 1C), then went through diplotene stage (Fig. 1C, 1 dpp), and finally arrested at the dictyate stage by 4 dpp (Fig. 1C). The timing of the cAMP increase and of meiotic progression suggests that the cAMP level could be involved in regulating meiotic development in perinatal mouse ovaries.

Given that intracellular cAMP is synthesized by adenylyl cyclases, we next determined the mRNA expression levels of all ten isoforms of mouse adenylyl cyclase in perinatal ovaries using qRT-PCR. Based on mRNA expression, *Adcy2* was likely to be the dominant isoform in perinatal ovaries (Fig. 2A). A significant increase in *Adcy2* mRNA expression was observed at 17.5 dpc, which was consistent with the change in cAMP levels in the perinatal ovary (Fig. 2A). Moreover, immunofluorescence staining for ADCY2 and the germline marker DEAD box polypeptide 4 (DDX4) demonstrated that the ADCY2 protein was present
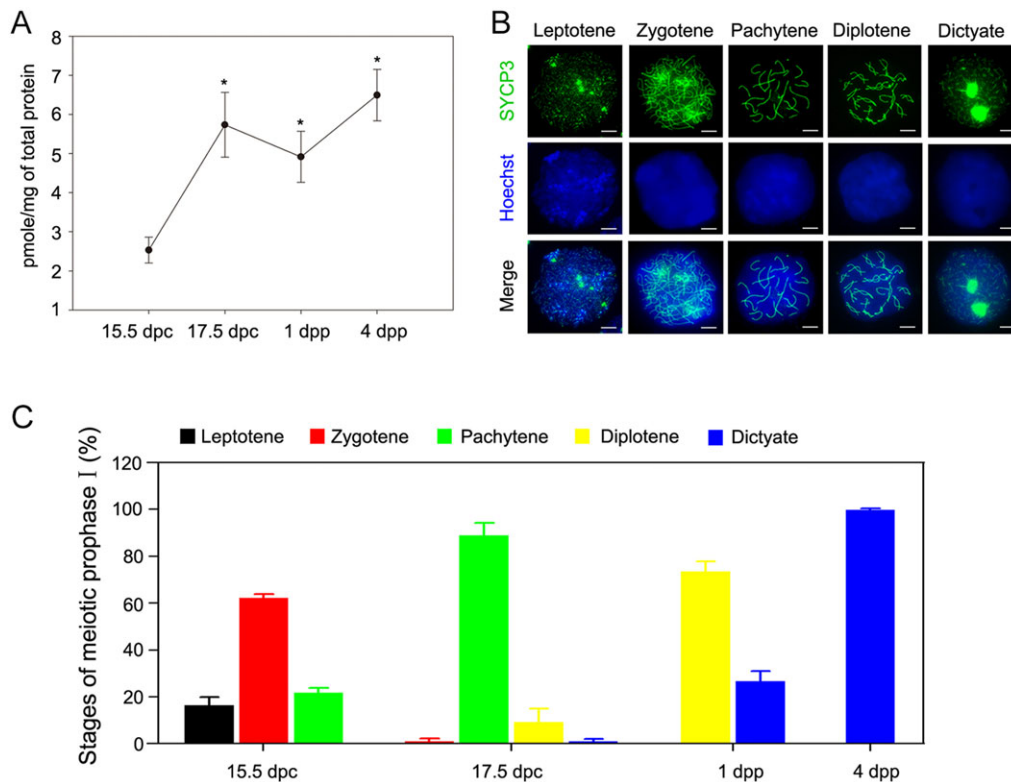
Fig. 1. The level of cAMP in oocytes correlates with progression through meiotic prophase I. (A) cAMP levels in perinatal mouse ovaries were measured at the indicated time points by radioimmunoassay. The level of cAMP increased significantly at 17.5 dpc and remained at a high levels until 4 dpp (*$P<0.05$; ANOVA and Holm–Šidák test). (B) Representative examples of the phases of meiosis prophase I in mouse oocytes. Nuclear spreads were immunolabeled for SYCP3 (green) and stained with the nuclear marker Hoechst (blue). The prophase stages were defined as: leptotene, a mass of fine threads of chromatin; zygotene, shorter and thicker chromosomes and classical tripartite synaptonemal complex structure at homologous pairing site; pachytene, maximal shortening and thickening of the paired homologous chromosomes; diplotene, separation of homologous chromosomes; dictyate, two to four nucleoli can be clearly seen and the chromosomes are decondensed and diffuse. Scale bars: 10 μm. (C) The profile of oocyte meiosis in perinatal ovaries at different time points. Oocytes at the zygotene stage are predominant in the ovary at 15.5 dpc. Most oocytes have developed to the pachytene stage at 17.5 dpc, then go through diplotene at 1 dpp, and are arrested at the dictyate stage at 4 dpp. All experiments were repeated at least three times, and more than 300 oocytes from two ovaries were analyzed for each time point.
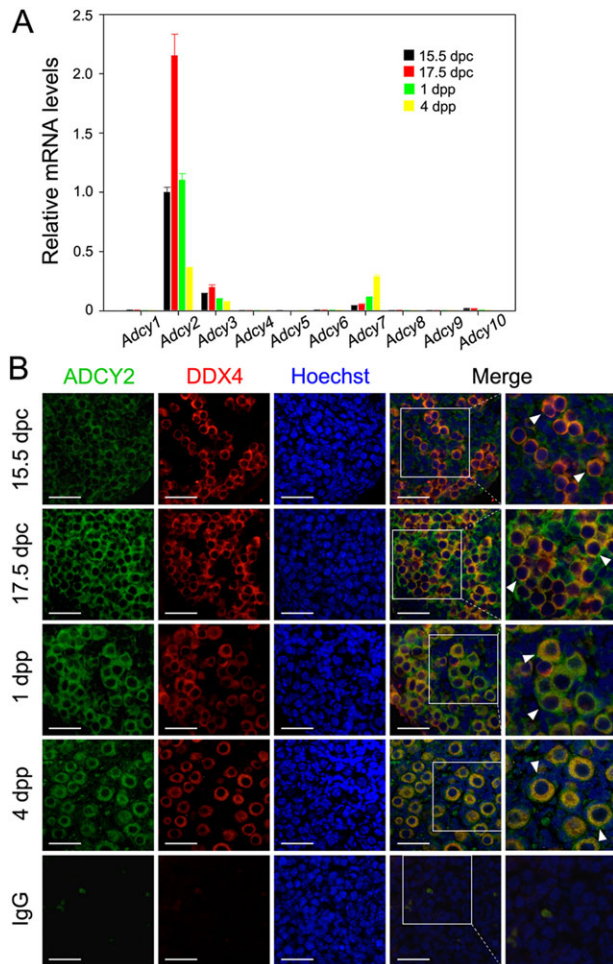
Fig. 2. Expression profile of adenylyl cyclases in perinatal ovaries.
(A) The mRNA expression of all ten isoforms of mouse adenylyl cyclase in fetal and neonatal ovaries was assessed by qRT-PCR. *Adcy2* was likely to be the dominant isoform in perinatal ovaries, and a significant increase in *Adcy2* expression was observed at 17.5 dpc. (B) Expression of ADCY2 protein in perinatal ovaries. Ovaries were stained for ADCY2 (green) and the oocyte marker DDX4 (red) at the indicated time points. ADCY2 protein was primarily detected in the oocytes of fetal and neonatal mouse ovaries (arrowheads). An isotype-matched IgG was used as the negative control. Scale bars: 50 µm.

primarily in oocytes and expressed at high levels in neonatal ovaries (Fig. 2B, arrowheads). The staining pattern of ADCY2 suggests that cAMP is likely to be predominantly produced by oocytes during early oogenesis.

Blocking the synthesis of cAMP suppresses oocyte meiosis prophase I in perinatal ovaries

To test the hypothesis that the increase in cAMP level is associated with early oocyte meiosis, we cultured fetal ovaries (16.5 dpc) with 3 µM MDL-12,330, an irreversible adenylyl cyclase inhibitor, for 2 or 4 days. After 2 days of *in vitro* culture, a high proportion (87.7±1.2%) of control oocytes were in the diplotene phase. By contrast, 2 days of MDL-12,330 treatment significantly delayed oocyte meiosis in the ovary, and only 48.3±2.9% of oocytes developed to the diplotene stage (Fig. 3A). After 4 days in culture, the majority (57.8±4.2%) of oocytes had developed to the dictyate stage in the control group (Fig. 3B), in contrast to only 19.8±8.2% after 4 days of *in vitro* culture with MDL-12,330, with most oocytes being blocked at the diplotene stage (Fig. 3B). The
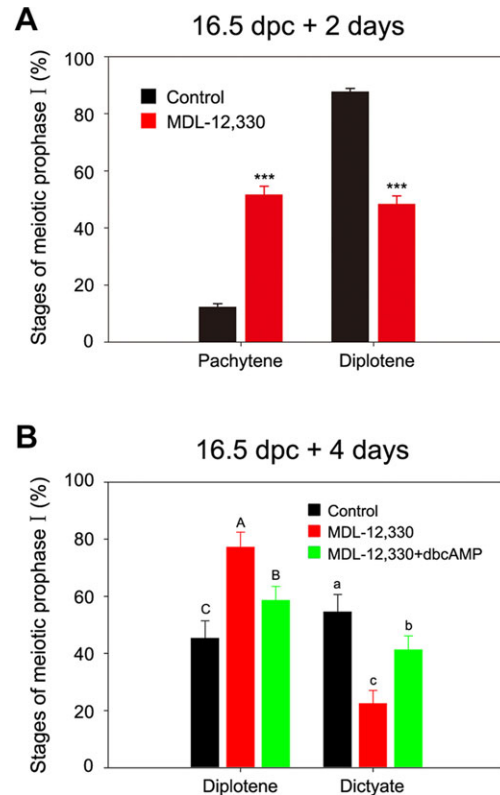
**Fig. 3. Blocking cAMP synthesis suppresses oocyte meiotic prophase I.**
Ovaries at 16.5 dpc were cultured in media alone (control) or with the irreversible adenylyl cyclase inhibitor MDL-12,330. Meiotic development was significantly delayed in MDL-12,330-treated ovaries after 2 days (A) and 4 days (B) of culture compared with the control group. The cAMP analog dbcAMP partially rescued the delayed meiotic development in the MDL-12,330-treated ovaries. ***$P<0.001$ (*t*-test), control versus treated ovaries. (B) Different letters (A-C, a-c) indicate significant differences between groups (ANOVA and Holm–Šidák test). B is significantly different from A and C. Similarly, b is significantly different from a and c.

MDL-12,330-mediated meiotic delay was significantly attenuated by adding dibutyryl cAMP (dbcAMP, 1 µM), an analog of cAMP, to the culture (Fig. 3B). Taken together, these results indicated that the cAMP level in oocytes regulates the progression of meiotic prophase I in perinatal mouse ovaries.

cAMP controls oocyte meiosis by regulating the degradation of SYCP1 during early oogenesis

A previous study reported that degradation of SYCP1 is essential for oocytes to enter the diplotene stage in rat neonatal ovaries (Paredes et al., 2005). Therefore, we examined whether the level of cAMP controls oocyte meiosis in perinatal mouse ovaries by regulating SYCP1.

To determine whether premature loss of SYCP1 accelerates entry into the diplotene stage, we knocked down SYCP1 using RNA interference (RNAi). Ovaries (16.5 dpc) were injected with *Sycp1*-specific small interfering RNA (siRNA) and cultured *in vitro* for 2 or 3 days. The levels of both *Sycp1* mRNA and SYCP1 protein decreased significantly after 2 days of siRNA treatment, indicating that knockdown of SYCP1 expression was highly efficient in culture (Fig. 4A,B). We then analyzed the meiotic progress of oocytes after 3 days of *Sycp1* siRNA treatment. The majority of the oocytes (67.0±4.2%) developed to the dictyate stage in the *Sycp1* siRNA-treated group, but only 31.7±6.3% of oocytes were at the
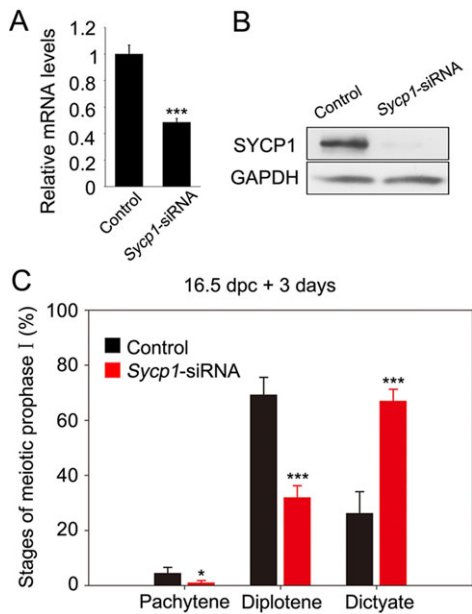
Fig. 4. Knockdown of SYCP1 expression accelerates entry into the diplotene and dictyate stages in cultured ovaries. (A,B) Validation of the SYCP1 knockdown efficiency. Ovaries at 16.5 dpc were injected with *Sycp1* siRNA or non-specific control siRNA. After 2 days of culture, both mRNA (A) and protein (B) levels of *Sycp1* were decreased in the *Sycp1* siRNA-treated group. (C) Knockdown of SYCP1 accelerated oocyte entry into the dictyate stage. Ovaries at 16.5 dpc were cultured for 3 days after siRNA treatment. The majority of the oocytes in the siRNA-injected ovaries developed to the dictyate stage; by contrast, most of the oocytes in the control group were in the diplotene stage. Asterisk indicates a significant difference between control and treated ovaries. *$P<0.05$, ***$P<0.001$ (*t*-test), control versus treated ovaries.

dictyate stage in the control group (Fig. 4C), demonstrating that suppressing SYCP1 expression accelerates meiotic development in the mouse fetal ovary.

We next examined whether the level of cAMP controls the disassembly and degradation of SYCP1 in oocytes. To assess the disassembly of the SYCP1 protein, we cultured ovaries (16.5 dpc) with MDL-12,330 and performed chromosome spreads and immunofluorescence staining as previously reported (Jordan et al., 2012). In this approach, linear SYCP1 staining indicated that the synaptonemal complex (SC) was integrated with SYCP1, whereas discontinuous or lack of SYCP1 staining indicated disassembly of SYCP1 from the SC (Fig. 5A). After 2 days of *in vitro* culture, the proportion of oocytes with linear SYCP1 staining was significantly higher in the MDL-12,330-treated ovaries than in the control group, indicating that disassembly of SYCP1 was inhibited by suppressing oocyte cAMP synthesis (Fig. 5B). SYCP1 degradation was also significantly suppressed in ovaries after 1 or 2 days of MDL-12,330 treatment beginning on 16.5 dpc (Fig. 5C). Adding 1 µM dbcAMP relieved the MDL-12,330-mediated suppression of SYCP1 degradation (Fig. 5D). Thus, the level of cAMP in the oocyte is likely to control the disassembly and degradation of SYCP1, a key SC protein of meiosis.

Suppression of cAMP synthesis disrupts germline cyst breakdown and primordial follicle formation

To investigate other potential mechanisms whereby the cAMP level in oocytes might control early oogenesis, we examined the expression levels of genes that have been reported to regulate early oogenesis, including *Cyp19a1*, *Kit*, *Kitl*, *Ctgf*, *Ngf* and components of the Notch pathway in MDL-12,330-treated fetal

ovaries by qRT-PCR (Fig. 6A). The expression of *Cyp19a1*, *Kit*, *Jag1*, *Hes1* and *Hey2* was significantly decreased after 4 days of MDL-12,330 treatment. By contrast, *Kitl*, *Ngf* and *Notch1*, which are mainly expressed in ovarian somatic cells (Guo et al., 2012; Pepling, 2012; Jones and Pepling, 2013), were increased in the MDL-12,330 treated ovaries (Fig. 6A). These results showed that the cAMP level in oocytes regulates the expression of a series of genes involved in primordial follicle formation, indicating that cAMP might play a functional role in controlling the formation of primordial follicle in mice.

We next investigated whether suppressing cAMP synthesis affects the formation of primordial follicles. The process of primordial follicle formation is initiated at 17.5 dpc with the breakdown of germline cysts, and concludes at ~5 dpp when the primordial follicle pool is established in the mouse ovary (Pepling, 2012). After 7 days of *in vitro* culture starting with 16.5 dpc fetal ovaries, most of the oocytes (5546±190, 72.5±2.4%; Fig. 6E) in the control group were surrounded by pregranulosa cells and had formed primordial follicles (Fig. 6B, arrowheads). By contrast, only 29.5±3.7% (2212±276) of the oocytes were surrounded by pregranulosa cells in MDL-12,330-treated ovaries; the majority of the oocytes (5025±643, 66.9±8.6%; Fig. 6E) were either naked or included in cysts that had not formed primordial follicles (Fig. 6C, arrows). dbcAMP (1 µM) was added to the cultures to confirm that cAMP levels contributed to regulating the formation of primordial follicles. As shown in Fig. 6E, dbcAMP rescued primordial follicle formation in the MDL-12,330/dbcAMP group, allowing the majority of oocytes (4410±706, 65.5±10.5%) to successfully form primordial follicles (Fig. 6D, arrowheads), at a proportion similar to the control and in contrast to the low proportion of primordial follicles formed in MDL-12,330-treated ovaries. The total number of oocytes in all of the groups was similar, indicating that oocyte survival was not affected by blocking cAMP (Fig. 6E). Taken together, our results indicated that the level of cAMP in oocytes plays a key role in controlling the formation of primordial follicles in the perinatal mouse ovary.

Dictyate arrest of oocytes is crucial for primordial follicle formation *in vivo*

Our *in vitro* studies indicated that the meiotic development of oocytes positively correlates with the formation of primordial follicles. To investigate this relationship under more physiological conditions, we compared oocyte meiosis and primordial follicle formation in neonatal ovaries at 1, 3 and 5 dpp. The proportion of oocytes in primordial follicles correlated with the percentage of oocytes in dictyate stage in neonatal ovaries *in vivo* (Table 1), suggesting that oocyte meiotic arrest might be related to the formation of primordial follicles *in vivo*.

To confirm our hypothesis, we used an *in situ* oocyte chromosome analysis approach. The ovaries were stained for the chromosome marker SYCP3 and the diplotene stage marker Y box protein 2 (MSY2; also known as YBX2). Using this approach, the stage of oocyte meiotic development in germline cysts or primordial follicles could be directly visualized *in situ* in a neonatal ovarian section (Fig. 7; supplementary material Fig. S1). At 1 dpp, 94.2± 2.6% of the oocytes were in germline cysts and most of the oocytes (72.6±3.2%) had developed to the diplotene stage, but had not entered the dictyate stage (Fig. 7A, arrows and arrowheads; Tables 1 and 2). Only 5.8±2.6% of the oocytes, which were located in the medullary region of the ovary, were surrounded by pregranulosa cells to form primordial follicles (Fig. 7B; Table 1). All of the oocytes in primordial follicles at 1 dpp were arrested at the dictyate
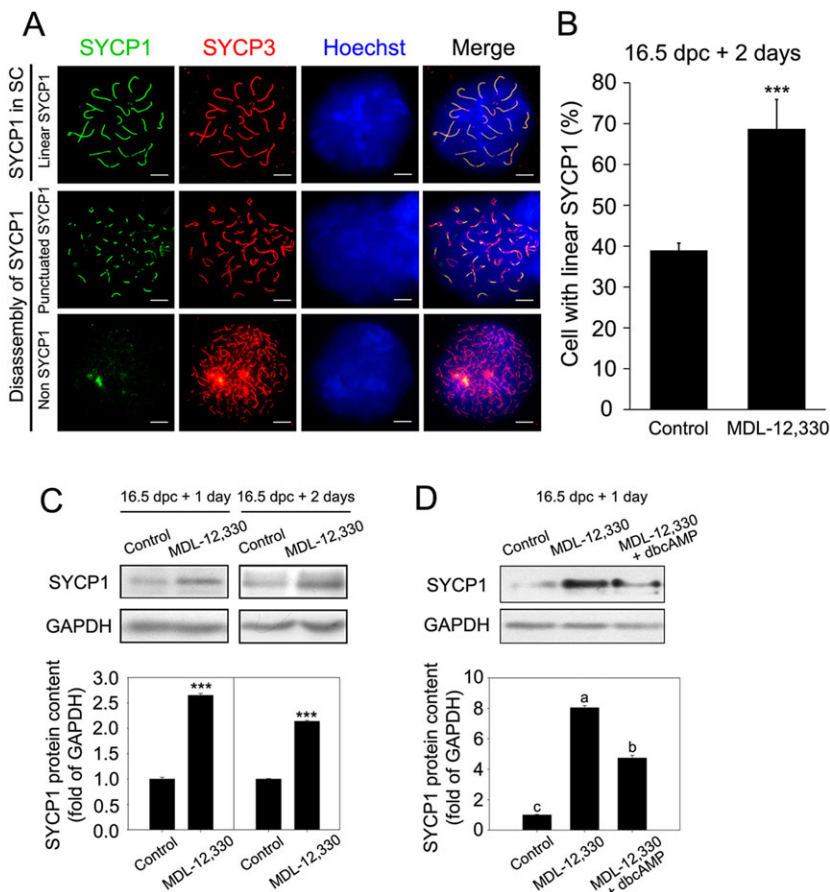
Fig. 5. Blocking cAMP synthesis disrupts the disassembly and degradation of SYCP1 in mouse perinatal ovaries. (A) SYCP1, which is a central synaptonemal complex (SC) component, and SYCP3, which is an SC lateral element component, were stained in oocytes from 17.5 dpc and 1 dpp ovaries. Linear SYCP1 staining indicated integrated assembly of SYCP1 in the oocyte SC, whereas punctuated SYCP1 or no SYCP1 staining indicated disassembly of the SYCP1 protein. (B) An increased percentage of oocytes with linear SYCP1 was observed in 16.5 dpc ovaries after 2 days of MDL-12,330 treatment. (C) SYCP1 degradation was suppressed after 1 or 2 days of MDL-12,330 treatment. ***$P<0.001$ (t-test), control versus treated ovaries. (D) The MDL-12,330-mediated suppression of SYCP1 degradation was rescued by addition of dbcAMP to the culture. Different letters (a-c) indicate significant differences between groups (ANOVA and Holm–Šidák test).

stage (Table 2; Fig. 7B, arrowheads; supplementary material Fig. S1). At 3 dpp, most of the oocytes (91.0±3.5%) had developed to the dictyate stage in the ovary. Only 9.0±3.5% of the oocytes were still in the diplotene stage, and those oocytes were in germline cysts localized in the cortical region of the ovary. All of the oocytes in primordial follicles were arrested at the dictyate stage of meiosis (Fig. 7C, arrowheads; Table 1). When the primordial follicle pool was fully established at 5 dpp, all of the oocytes were arrested at the dictyate stage, and the ovary did not contain any oocytes at earlier meiotic stages (Fig. 7D, arrowheads). Thus, dictyate stage arrest of oocytes is essential for forming primordial follicles *in vivo*.

DISCUSSION
Our results reveal the functional role of oocyte cAMP in controlling early oogenesis and folliculogenesis in the perinatal mouse ovary. Several convergent lines of evidence demonstrate that cAMP levels in oocytes regulate the progression of meiotic prophase I, probably by affecting the disassembly and degradation of SYCP1 in the oocyte. In addition, the appropriate level of oocyte cAMP is essential for primordial follicle formation in mice.

As a second messenger, cAMP regulates many cellular responses and orchestrates a network of intracellular events (Guellich et al., 2014). In the reproductive system, cAMP has been reported to regulate the proliferation of mouse PGCs (De Felici et al., 1993) and folliculogenesis in cultured human ovarian cortical pieces (Zhang et al., 2004). Moreover, high levels of cAMP are essential to maintain the meiotic arrest of immature oocytes in mammals (Conti et al., 2012). However, it was not clear whether cAMP contributes to early oocyte meiosis and oogenesis and, if so, what the mechanism of cAMP involvement might be. In this study, we have demonstrated

that cAMP is pivotal for early meiosis and oogenesis. Our results suggested that increased cAMP in the fetal ovary enhances oocyte progression to the dictyate stage of meiosis. In addition, the meiotic arrest of immature oocytes is maintained in adults by consistently high levels of intra-oocyte cAMP (Conti et al., 2012). We conclude that cAMP is indispensable throughout the process of female germ cell development in mice, and that the oocyte cAMP level plays a vital role in maintaining proper female reproduction in mammals.

Previous studies demonstrated that separation of the SC is essential for spermatocytes to enter the diplotene stage of meiosis in rodents (Tarsounas et al., 1999; Jordan et al., 2012). Here we show that the level of oocyte cAMP regulates the dissolution of SYCP1 from the SC. SYCP1 is a core component of the SC (Yang and Wang, 2009; Fraune et al., 2012) and the disassembly of SYCP1 is a key step to entering the diplotene stage in rat oocytes (Paredes et al., 2005). Thus, we hypothesize that the level of cAMP in oocytes potentially controls the progress of meiotic prophase I by regulating SC separation in the perinatal ovary. However, additional studies are required to understand the events downstream of cAMP that regulate the process of oocyte meiosis.

It has long been thought that the progression of oocyte meiosis is coordinated with the formation of follicles in the fetal mouse ovary (Slizynski, 1957). Previous studies using genetically modified mouse models reported that deleting meiotic prophase I-related genes results in abnormal meiotic development in oocytes and a failure to establish the primordial follicle pool in the ovary (Pepling, 2006; Edson et al., 2009). These reports support the contention that there is a correlation between meiotic development and the formation of primordial follicles in the ovary. Consistent with these studies, when we used RNAi to knockdown SYCP1 in
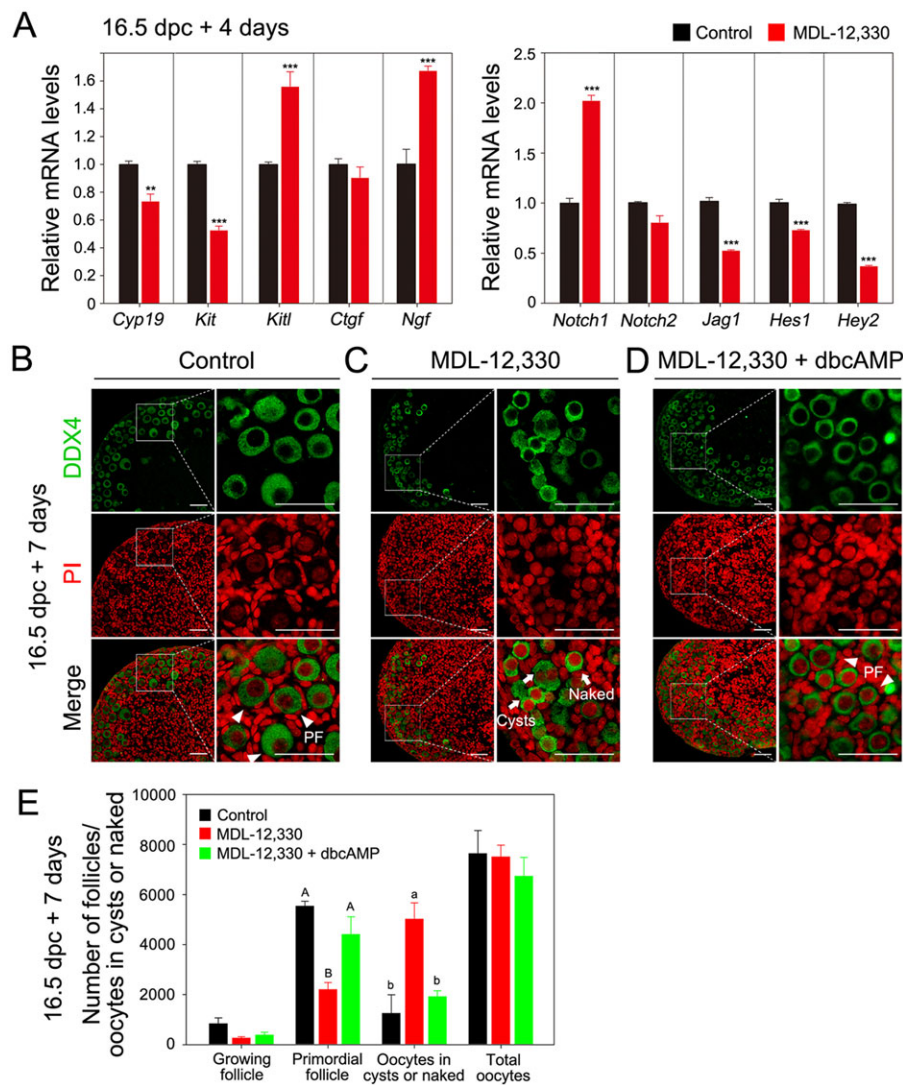
Fig. 6. Blocking cAMP synthesis disrupts oogenesis and primordial folliculogenesis in fetal ovaries. Ovaries at 16.5 dpc were cultured with or without MDL-12,330 and dbcAMP for 4 days or 7 days *in vitro*. (A) cAMP regulated the expression of oogenesis and primordial folliculogenesis genes as detected by qRT-PCR after 4 days in culture with MDL-12,330. The expression of *Kitl*, *Ngf* and *Notch1* increased, whereas *Cyp19a1*, *Kit*, *Jag1*, *Hes1* and *Hey2* decreased in the ovary. Asterisk indicates a significant difference between control and treated ovaries. **P<0.01, ***P<0.001 (*t*-test), control versus treated ovaries. (B-D) Blocking cAMP synthesis disrupted germline cyst breakdown and primordial follicle formation. After 7 days in culture, sections of ovary were immunolabeled for the oocyte marker DDX4 (green) and stained with the nuclear marker propidium iodide (PI, red). In the control group, a normal distribution of primordial follicles (arrowheads) was observed (B). Most of the oocytes were in cysts or naked (arrows) in the MDL-12,330-treated group (C). Comparable primordial follicle formation (arrowheads) was observed in control and MDL-12,330/dbcAMP groups (D). PF, primordial follicle. Scale bars: 50 μm. (E) Quantifying the numbers of oocytes in the ovaries of the different treatment groups. A significant reduction in primordial follicle formation was observed in the MDL-12,330-treated ovaries and the formation of primordial follicles was rescued by adding dbcAMP. The total numbers of oocytes were similar between groups. The different letters (A,B, a,b) indicate significant differences between groups (ANOVA and Holm–Šidák test).

mice, primordial follicle formation was accelerated at 1 dpp (supplementary material Fig. S2). We also provide evidence that oocyte cAMP levels play an essential role in controlling the progression of meiotic prophase I in oocytes and the formation of primordial follicles in the perinatal ovary by regulating the expression of several crucial genes or proteins. Therefore, we conclude that cAMP is key regulator of early oogenesis in the perinatal mammalian ovary.

A pioneering study proposed the 'production line' hypothesis that the temporal order of germ cells entering meiosis determines the temporal order of oocytes activated for ovulation as an adult (Henderson and Edwards, 1968). Using a radioactive labeling approach and an *in vivo* transplantation model, Polani and Crolla demonstrated that the temporal order of oocyte maturation in the mouse ovary is related to the temporal order of entry into meiosis during the fetal stage (Polani and Crolla, 1991). In the current study, we provide direct evidence that meiotic arrest of oocytes in the dictyate stage is essential for the formation of primordial follicles in the mouse ovary and therefore our results also support the production line hypothesis that the temporal order of oocyte meiotic arrest is correlated to the temporal order of primordial follicle formation *in vivo*.

Recently, using a *Stra8*-deficient mouse model Dokshin et al. reported that some female germ cells form follicles in the absence of meiotic entry (Dokshin et al., 2013). However, the majority of the germ cells in the *Stra8*-deficient ovary failed to form primordial follicles at birth, and the ovary was devoid of germ cells by 6 to 8 weeks of age (Dokshin et al., 2013). In our study, we provide direct evidence that oocyte meiotic arrest in the dictyate stage is essential for the formation of primordial follicles in the mouse ovary, and that oocyte cAMP levels regulate this process. Therefore, we conclude that proper oocyte meiosis is essential for primordial follicle formation under physiological conditions.

In conclusion, our results indicate that oocyte-derived cAMP is important for early oogenesis and folliculogenesis in mice. These findings could contribute to opening new avenues of research to expand our understanding of physiological and pathological processes in the mammalian ovary.

MATERIALS AND METHODS
Animals
All CD1 mice were purchased from the Laboratory Animal Center of the Institute of Genetics and Developmental Biology (Beijing, China). Female mice at 6-8 weeks of age were caged with males at a ratio of 1:1 overnight
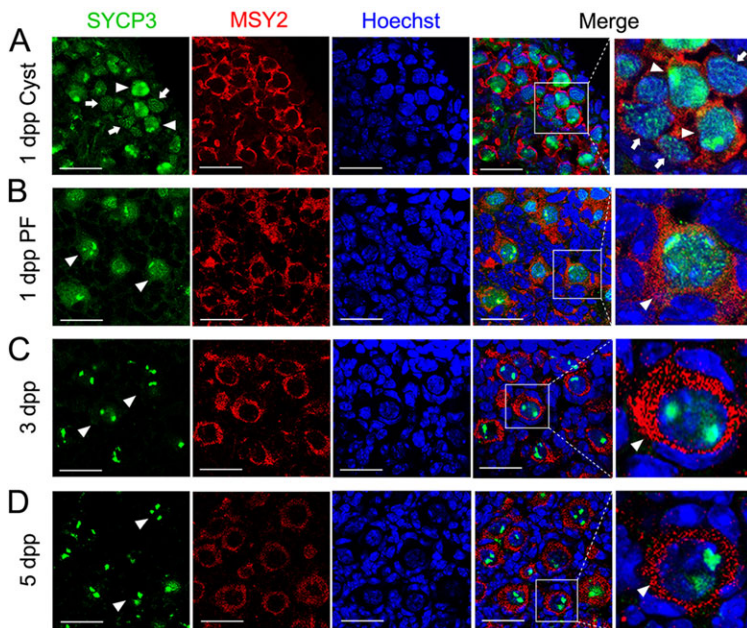
Fig. 7. Oocyte arrest at dictyate stage is crucial for primordial follicle formation. Ovaries were *in situ* immunolabeled for SYCP3 (green) to investigate oocyte meiosis, and the diplotene marker MSY2 (red) was used to identify germline cells. (A) The majority of the oocytes in the ovarian cortical region at 1 dpp were in cysts but had not formed primordial follicles, and most were still in the diplotene stage. Arrows indicate diplotene stage and arrowheads indicate dictyate stage. (B) All of the oocytes in 1 dpp ovaries that had formed primordial follicles were located in the medullary region and were arrested at the dictyate stage (arrowheads). (C,D) All of the oocytes in the primordial follicles at 3 dpp and 5 dpp were arrested at the dictyate stage (arrowheads). Scale bars: 50 µm.

and checked for a vaginal plug the following morning. The presence of a vaginal plug was considered 0.5 dpc [also known as embryonic day (E)]. The day the pups were born was considered 0.5 dpp [also known as postnatal day (P)]. All mice were housed at China Agricultural University under 16/8 h light/dark cycles at 26°C with access to chow and water ad libitum. The animal experiments conformed to the guidelines and regulatory standards of the Institutional Animal Care and Use Committee of China Agricultural University.

Ovary isolation and culture
Ovaries were separated by microdissection from the mesonephros or ovarian capsule in pre-chilled PBS (10 mM, pH 7.4) under a stereomicroscope. The isolated ovaries were cultured on an insert (PICM0RG50, Millipore) in 6-well culture dishes (NEST Biotechnology) with 1.2 ml basic DMEM/F-12 medium (Gibco, Life Technologies) at 37°C in a 5% CO_2, 95% air atmosphere with saturated humidity.

To assess the role of cAMP in oocyte meiosis, 16.5 dpc ovaries (two ovaries/group) were cultured for 1-4 days in either medium alone or in medium supplemented with MDL-12,330 (M182, Sigma-Aldrich). To determine the role of cAMP in primordial follicle formation, we cultured 16.5 dpc ovaries (five ovaries/group) for 7 days with or without MDL-12,330. dbcAMP (D0627, Sigma-Aldrich) was used for rescue experiments.

Radioimmunoassay (RIA)
The amount of cAMP present was measured by RIA. Ovaries were collected at 15.5 dpc, 17.5 dpc, 1 dpp and 4 dpp. Groups of 20-30 ovaries were mechanically dissociated by manual pipetting in 100 µl 0.1 M HCl, and the solution was snap-frozen in liquid nitrogen after solubilizing the ovaries on ice for at least 10 min. The ovaries were transferred and stored at −80°C. For the RIA, the samples were thawed, centrifuged at 12,000 *g* for 5 min, the supernatant collected and then dried overnight at 60°C. The cAMP RIA

(IM117, Immunotech) had a sensitivity of 0.2 nM cAMP, an intra-assay coefficient of variation (CV) of 11%, and an interassay CV of 16%. A standard curve was constructed using a log-linear curve fit with B/B_o (%) (*y*-axis, where B is the average cpm of the paired standards and B_o is the cpm of total activity) against cAMP concentration (*x*-axis). Values were normalized to the amount of protein (mg) present in the sample. The protein content was measured using the BCA protein assay kit (CellChip Beijing Biotechnology Company) using bovine serum albumin (BSA) as the standard.

Histological sections and follicle counts
Ovaries were fixed in cold 4% paraformaldehyde (PFA) for 24 h, embedded in paraffin, and serially sectioned at 5 µm. The sections were stained with Hematoxylin, and the numbers of oocytes and follicles were counted in every fifth section; to estimate the total numbers of oocytes and follicles in each ovary, the sum was multiplied by five.

Chromosome spreads and immunofluorescence staining
We used a SYCP3 antibody to identify the chromosomal axial elements at meiosis prophase I, as described previously (Mu et al., 2013). Combined staining for SYCP1 and SYCP3 was used to determine whether SYCP1 disassembly occurred. The stages of meiotic prophase I were evaluated based on the appearance of axial elements according to previous studies (Hartung et al., 1979; Prieto et al., 2004). In total, 300 oocytes from two ovaries were counted on each slide, and repeated for three animals. The primary antibodies and dilutions used are presented in supplementary material Table S1.

qRT-PCR
mRNA was extracted from ten ovaries for each sample using TRIzol Reagent (Invitrogen, Life Technologies), according to the manufacturer's protocol. The quantity and quality of the total RNA were determined using a Nanodrop (Thermo Scientific). Reverse transcription (Promega Reverse Transcription System) was performed using 1 µg total RNA per sample.

Table 1. The relationship between meiosis prophase I arrest and primordial follicle formation in 1 dpp and 3 dpp ovaries

Stage	Diplotene (%)	Dictyate (%)	Oocytes in primordial follicles (%)
1 dpp	73.5±4.3	26.6±4.3	5.8±2.6
3 dpp	9.0±3.5	91.0±3.5	75.5±6.4

Results are expressed as the percentage of total oocytes at a given stage (mean±s.d.).

Table 2. Progress of meiosis prophase I in 1 dpp ovaries

Progress	Diplotene (%)	Dictyate (%)
Oocyte in cysts	72.6±3.2	27.3±3.2
Oocyte in primordial follicles	1.0±1.0	99.0±1.0

Results are expressed as the percentage of total oocytes at a given stage (mean±s.d.).

Gene expression changes were analyzed by qRT-PCR in 96-well plates (Applied Biosystems) in 15 µl reaction volumes and normalized to *Gapdh*. The PCR was performed on an ABI 7500 Sequence Detection System (Applied Biosystems) using the following parameters: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Primers are listed in supplementary material Table S2. A melting curve was generated at increments of 0.5°C every two cycles (62 cycles total) starting at 65°C, with fluorescence acquired after each step.

Immunofluorescence labeling and *in situ* oocyte chromosome analysis

Ovaries were fixed in cold 4% PFA for 24 h, embedded in paraffin, and serially sectioned at 5 µm for immunofluorescence labeling and 10 µm for *in situ* oocyte chromosome analysis. The sections were deparaffinized, rehydrated, and subjected to high temperature (95-98°C) antigen retrieval with 0.01% sodium citrate buffer (pH 6.0). The sections were then blocked with ADB [3% BSA, 1% normal donkey serum in TBS (0.05 M Tris-HCl pH 7.6 and 0.15 M NaCl)] and incubated with primary antibodies overnight at 37°C before staining. Primary antibodies and dilutions used are presented in supplementary material Table S1. After rinsing thoroughly with PBS, the sections were incubated with Alexa Fluor 488- or 555-conjugated secondary antibodies (1:100, Invitrogen) at 37°C for 70 min in ADB. The sections were then rinsed with PBS and stained with Hoechst 33342 (B2261, Sigma) for 15 min. Finally, 20 µl Vectashield mounting medium (Applygen) was applied to each slide, and a coverslip was sealed in place. A Nikon 80i was used for imaging immunofluorescent sections and an Olympus FV100 was used for imaging *in situ* oocyte chromosome analysis. An isotype-matched IgG was used as the negative control.

RNAi knockdown in fetal ovaries

Ovarian RNAi was undertaken as described previously (Guo et al., 2012). Ovaries at 16.5 dpc were injected with 0.5 µl 20 µM siRNAs (Genepharma) using a glass pipette. Once the ovaries were full of liquid, electrotransfection was performed by applying three 5-ms quasi-square pulses at a pulse field strength of up to 40 V/cm. Total mRNA and protein were extracted from the ovary 48 h after injection to determine the effect of RNAi. *Sycp1* siRNA was 5′-TGCTGAAGAATTACAAGAGAAA-3′, targeting nucleotides 1488-1509 of the *Sycp1* mRNA. The non-targeting control siRNA was 5′-ACGTGACACGTTCGGAGAATT-3′, which has no homology with any known mouse mRNA.

Immunoblotting

Western blot analyses were conducted as described previously (Mu et al., 2013). Briefly, total proteins were extracted in WIP (CellChip Beijing Biotechnology Company) according to the manufacturer's protocol. The BCA protein assay kit was used to measure protein concentration. Electrophoresis was performed with 50 µg total proteins separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (IPVH00010, Millipore). The membranes were incubated overnight at 4°C with anti-SYCP1 antibody, which detected a 111 kDa band. The secondary antibody (ZB-2301, ZB-2305 from ZSGB-BIO, Beijing, China) was diluted 1:5000 in TBST (TBS plus 0.5% Tween 20). The membranes were visualized using the SuperSignal chemiluminescent detection system (34080, Thermo Scientific). GAPDH was used as an internal control. An Alpha Imager 2200 was used to quantify the relative amount of protein. Primary antibodies and dilutions used are listed in supplementary material Table S1.

Statistical analysis

All of the culture and immunofluorescence analyses were repeated at least three times using ovaries from different fetuses. Data are presented as mean±s.d. with each experiment performed in triplicate. Two ovaries per group were used for chromosome spreading and three to five were used for germ cell counting. Data were analyzed by *t*-test or analysis of variance (ANOVA). When a significant *F* ratio was detected by ANOVA, the groups were compared using the Holm–Šidák test. Data were considered statistically significant at $P<0.05$.

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