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RESEARCH ARTICLE

JNK signaling regulates E-cadherin junctions in germline cysts and determines primordial follicle formation in mice

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ABSTRACT

Physiologically, the size of the primordial follicle pool determines the reproductive lifespan of female mammals, while its establishment largely depends on a process of germline cyst breakdown during the perinatal period. The mechanisms regulating this process are poorly understood. Here we demonstrate that c-Jun amino-terminal kinase (JNK) signaling is crucial for germline cyst breakdown and primordial follicle formation. JNK was specifically localized in oocytes and its activity increased as germline cyst breakdown progressed. Importantly, disruption of JNK signaling with a specific inhibitor (SP600125) or knockdown technology (Lenti-JNK-shRNAs) resulted in significantly suppressed cyst breakdown and primordial follicle formation in cultured mouse ovaries. Our results show that E-cadherin is intensely expressed in germline cysts, and that its decline is necessary for oocyte release from the cyst. However, inhibition of JNK signaling leads to aberrantly enhanced localization of E-cadherin at oocyte-oocyte contact sites. WNT4 expression is upregulated after SP600125 treatment. Additionally, similar to the effect of SP600125 treatment, WNT4 overexpression delays cyst breakdown and is accompanied by abnormal E-cadherin expression patterns. In conclusion, our results suggest that JNK signaling, which is inversely correlated with WNT4, plays an important role in perinatal germline cyst breakdown and primordial follicle formation by regulating E-cadherin junctions between oocytes in mouse ovaries.

KEY WORDS: JNK, E-cadherin, Cadherin 1, MAPK, WNT4, Germline cyst, Primordial follicle

INTRODUCTION

The reproductive lifespan of female mammals is determined at the time of birth through the establishment of the pool of primordial follicles, which comprise arrested oocytes enclosed by a layer of flattened pre-granulosa cells (Faddy et al., 1992; Kezele et al., 2002). Mammalian females are incapable of producing oocytes and follicles after birth, and hence the oocytes in the primordial follicles represent the entire available reproductive source (Zhang et al., 2014, 2012). Any aberration in the formation of primordial follicles is likely to result in infertility (Xu and Gridley, 2013); however, the mechanisms regulating this process remain unknown.

In mice, primordial germ cells (PGCs), which are called oogonia in females, reach the urogenital ridges at ∼11 days post-coitum (dpc). Subsequently, the oogonia initiate rapid mitotic division and form germline cysts accompanied by incomplete cytokinesis and an intercellular bridge connection (Ginsburg et al., 1990; Pepling, 2012). After the oocytes progress through leptotene, zygotene, pachytene, diplotene and arrest at the dictyate stage of meiotic prophase I, the germline cysts undergo programmed breakdown between 17.5 dpc and 4 days post-partum (dpp), during which time approximately one-third of the oocytes survive to form primordial follicles (Pepling and Spradling, 2001; Wang et al., 2014). Accurate regulation of germline cyst breakdown during embryonic development is crucial to female fertility (Xu and Gridley, 2013). The c-Jun amino-terminal kinase (JNK) signaling pathway is a candidate for participating in controlling cyst breakdown, since it is known to be important in germ cell survival and follicle progression at several developmental stages (Bagowski et al., 2001; Etchegaray et al., 2012; Oktem et al., 2011).

The JNK signaling cascade is evolutionarily conserved and widely known for its role in regulating cell proliferation, migration and apoptosis (Deng et al., 2003; Huang et al., 2003; Pallavi et al., 2012). JNK kinases belong to the mitogen-activated protein kinase (MAPK) group of serine/threonine protein kinases. In mammalian cells, the JNK subfamily consists of three isoforms: JNK1, JNK2 and JNK3 (also known as MAPK8, MAPK9 and MAPK10). JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is specifically expressed in the brain, heart and testes (Chang and Karin, 2001). JNK was recently found to regulate adherens junction formation. The inhibition of JNK kinase activity promoted the localization of the E-cadherin–β-catenin complex to cell-cell contact sites (Lee et al., 2009, 2011). In addition to its action in mammalian cells, JNK activation has been associated with downregulation of the E-cadherin–β-catenin complex and a loss of cell polarity in *Drosophila* epithelial cells (Igaki et al., 2006).

The JNK pathway is involved in various ovarian developmental processes. In *Xenopus*, JNK signaling plays an essential role in controlling meiotic resumption in oocytes (Bagowski et al., 2001). Activated JNK signaling is required for the removal of dead follicular cells in the *Drosophila melanogaster* ovary (Etchegaray et al., 2012). JNK was also reported to regulate granulosa cell proliferation in rat ovaries (Oktem et al., 2011). Despite studies examining several ovarian developmental stages, it is currently unclear whether JNK signaling plays a role in cyst breakdown and primordial follicle formation, as well as its potential mechanism of involvement.

Here we investigated the expression and function of JNK signaling in perinatal mouse ovary development. Our data reveal that JNK signaling inhibition significantly suppresses the breakdown of germline cysts and that this process is associated with a rearrangement of E-cadherin-meditated oocyte adhesion. Moreover, evidence suggests that WNT4 plays a pivotal role in JNK pathway-mediated primordial follicle formation.

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RESULTS

JNK signaling inhibition suppresses primordial follicle formation

To determine whether JNK signaling is involved in germline cyst breakdown and primordial follicle formation, we first examined the cellular localization and expression pattern of JNK. We labeled sections of 17.5 dpc, 19.5 dpc, 2 dpp and 4 dpp ovaries with an antibody against JNK, as well as the oocyte marker DDX4. JNK was expressed at high levels during this period, primarily in oocyte cytoplasm (Fig. 1A). Western blotting revealed that total JNK levels were similar but that the phosphorylated form (P-JNK) varied distinctly in ovaries of different developmental stages. P-JNK was present at low levels at 17.5 dpc, but gradually increased from 19.5 dpc to 4 dpp (Fig. 1B). As active JNK was reported to participate in the formation and development of various organ systems (Dush and Nascone-Yoder, 2013; Zhuang et al., 2006), the pattern of P-JNK abundance implied that JNK signaling might participate in perinatal mouse ovary development.

Primordial follicle formation begins at 17.5 dpc, as marked by cyst breakdown, and finishes at ~4 dpp (Pepling, 2012). To investigate the consequences of JNK signaling inhibition during this period, we utilized SP600125, a JNK inhibitor. Western blotting demonstrated that 5 µM SP600125 could significantly inhibit c-JUN phosphorylation at Ser63 when 17.5 dpc ovaries were cultured with SP600125 for 3 days (Fig. 1C), which proved the efficacy of the inhibitor. Next, ovaries at 17.5 dpc were cultured *in vitro* with dimethylsulfoxide (DMSO, as a control) or 5 µM SP600125 for 6 days. A morphological comparison showed that the majority of the oocytes in the control ovaries became surrounded by pre-granulosa cells and formed primordial follicles (Fig. 1D,E, arrows). Conversely, SP600125-treated ovaries contained huge tracts of oocytes remaining within the cysts (Fig. 1D,E, arrowheads). Moreover, SP600125-treated ovaries contained fewer growing follicles than control ovaries (data not shown).

Knockdown of JNK1/2 expression suppresses primordial follicle formation

Given that SP600125 cannot distinguish between JNK1- and JNK2-mediated effects, lentivirus constructs expressing JNK1 or JNK2 shRNAs (Lenti-JNK1-sh1, Lenti-JNK1-sh2, Lenti-JNK2-sh1 and Lenti-JNK2-sh2) were designed to individually target and knockdown JNK1 and JNK2 expression in the fetal mouse ovary; the lentivirus expressing a scramble sequence of shRNA was used as a control. After 3 days of lentivirus transfection, strong green fluorescence was observed by fluorescence microscopy (Fig. 2A) and mRNA levels of endogenous *Jnk1* and *Jnk2* were efficiently downregulated (Fig. 2B). The morphology of the ovaries was analyzed after 6 days of culture. Similar to the effect of SP600125 treatment, significantly more oocytes remained in the cysts of ovaries transfected with Lenti-JNK1-sh2, Lenti-JNK2-sh1, or Lenti-JNK1-sh2 plus Lenti-JNK2-sh1 compared with control ovaries, and the cyst breakdown failure appeared most severe

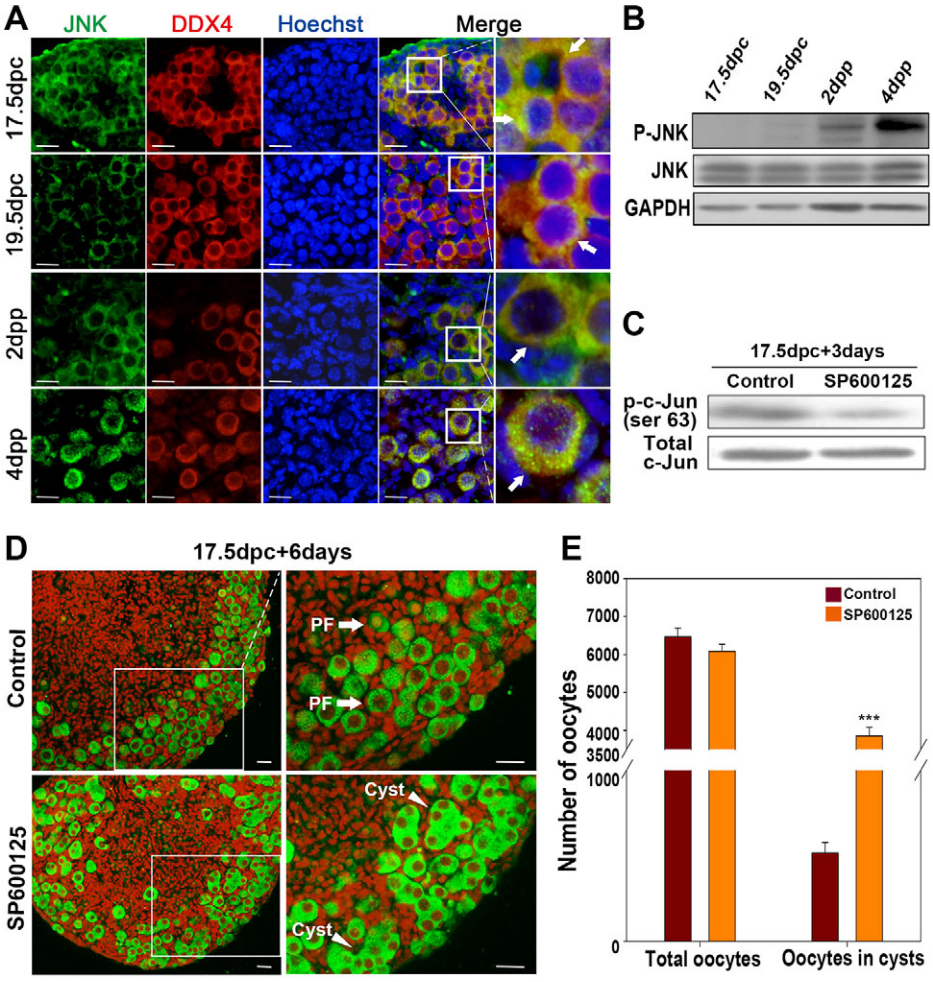

Fig. 1. JNK signaling inhibition suppresses primordial follicle formation. (A) Cellular localization of JNK in perinatal ovaries. Ovaries were stained for JNK and the oocyte marker DDX4 at 17.5 dpc, 19.5 dpc, 2 dpp and 4 dpp. Hoechst was used to identify nuclear DNA. JNK was primarily localized in the cytoplasm of oocytes of perinatal mouse ovaries (arrows). (B) Western blotting analysis of JNK and P-JNK levels in the mouse ovaries from 17.5 dpc to 4 dpp. GAPDH was used for normalization. (C-E) 17.5 dpc ovaries were cultured with SP600125 for 3 days or 6 days *in vitro*. (C) Western blotting was performed to examine the efficacy of the inhibitor. 5 µM SP600125 significantly inhibited the phosphorylation of c-JUN at Ser63 after 3 days of culture. (D) JNK signaling attenuation disrupted germline cyst breakdown. After 6 days of culture, ovarian sections were immunolabeled for DDX4 (green) and stained with the nuclear marker propidium iodide (PI, red). In the control group, most of the oocytes were surrounded by pre-granulosa cells and formed primordial follicles (arrows). By contrast, SP600125-treated ovaries showed huge tracts of oocytes remaining within the cysts (arrowheads). PF: Primordial follicles (PF) contain an oocyte surrounded by a single layer of pre-granulosa cells with flattened nuclei. (E) Total numbers of oocytes were similar between the groups. SP600125-treated ovaries contained significantly more oocytes within cysts. ***P<0.001 (t-test), control versus SP600125-treated ovaries. Scale bars: 25 µm.

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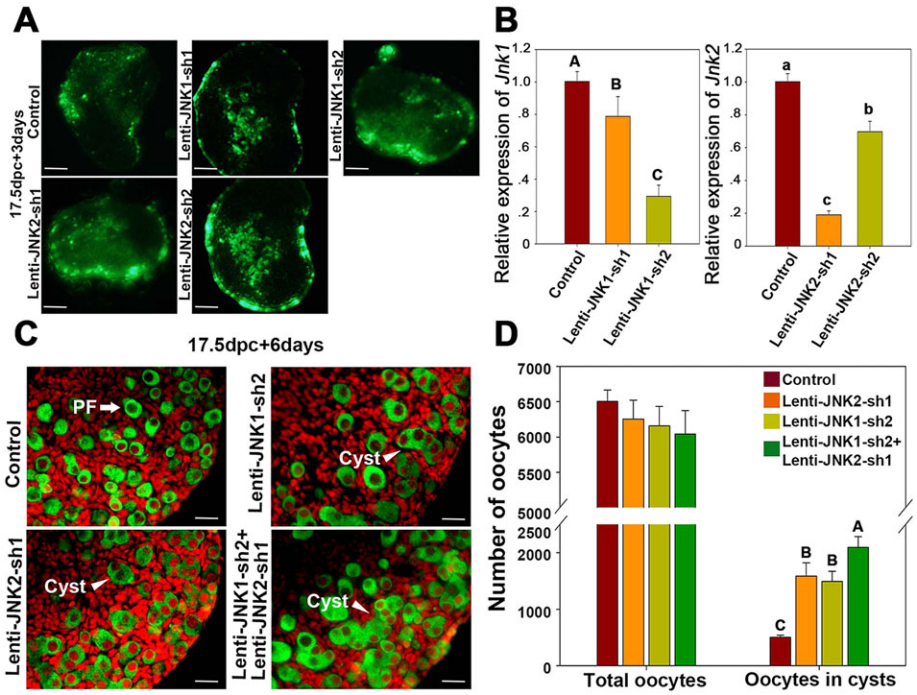

Fig. 2. Knockdown of JNK1/2 expression disrupts primordial folliculogenesis. 17.5 dpc ovaries were transfected with control lentivirus or Lenti-JNK-shRNAs for 3 days or 6 days *in vitro*. (A) Fluorescence microscopy analysis of lentivirus infection efficiency. Strong green fluorescence was observed following 3 days of lentivirus infection. Scale bars: 100 µm. (B) qRT-PCR analysis of JNK1/2 knockdown efficiency. Endogenous *Jnk1* and *Jnk2* mRNA expression was clearly downregulated in Lenti-JNK-shRNA-transfected ovaries after 3 days of culture. (C) Downregulation of JNK1/2 expression led to a failure of cyst breakdown. After 6 days of culture, ovaries transfected with Lenti-JNK-shRNAs exhibited many oocytes remaining within cysts (arrowheads). (D) Lenti-JNK-shRNA-transfected ovaries contained more oocytes within cysts compared with the control group. Different letters within bar charts (A-C, a-c) indicate significant differences between groups (ANOVA and Holm-Sidak test). Scale bars: 25 µm.

in the Lenti-JNK1-sh2 plus Lenti-JNK2-sh1 group (Fig. 2C,D). In summary, our results indicate that JNK signaling is essential for normal germline cyst breakdown and primordial follicle formation.

JNK inhibition results in aberrant E-cadherin junctions

To determine the molecular mechanisms by which JNK signaling might control cyst breakdown and primordial follicle formation, the expression levels of genes known to be involved in primordial

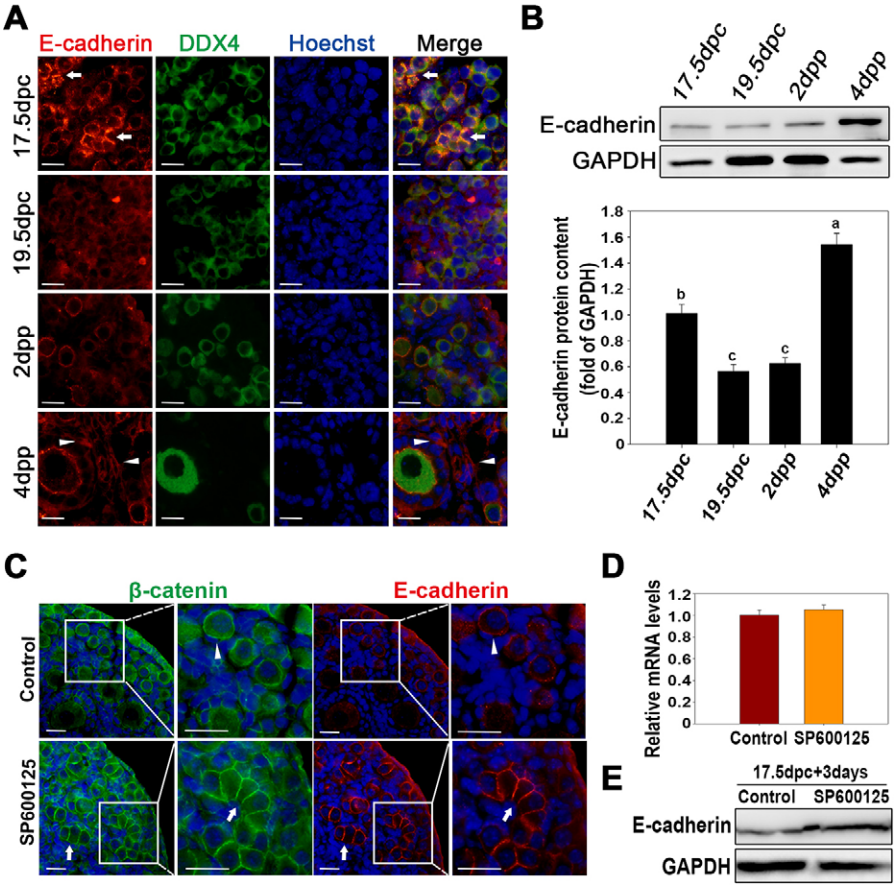

Fig. 3. JNK inhibition results in aberrant E-cadherin junctions. (A) Cellular localization of E-cadherin in perinatal ovaries. Ovaries were stained for E-cadherin and the oocyte marker DDX4 at 17.5 dpc, 19.5 dpc, 2 dpp and 4 dpp. Hoechst was used to identify nuclear DNA. E-cadherin was intensely detected at oocyte-oocyte contact sites inside cysts of 17.5 dpc ovaries (arrows), exhibited diffuse and weak staining in 19.5 dpc and 2 dpp ovaries, and localized in oocytes and somatic cells (arrowheads) of 4 dpp ovaries. (B) Western blotting analysis of E-cadherin expression patterns in the mouse ovaries from 17.5 dpc to 4 dpp. GAPDH was used for normalization. Expression of E-cadherin followed a V-shape pattern. Different letters (a-c) indicate significant differences between groups (ANOVA and Holm-Sidak test). (C) 17.5 dpc ovaries were cultured with SP600125 for 6 days *in vitro* and immunofluorescence was performed to examine the E-cadherin junctions. Attenuation of JNK signaling enhanced the localization of E-cadherin and β-catenin at oocyte-oocyte contact sites inside the cysts (arrows), whereas expression of E-cadherin and β-catenin was weak in the control group (arrowheads). (D,E) Ovaries at 17.5 dpc were treated with 5 µM SP600125 for 3 days *in vitro*. (D) qRT-PCR analysis showed no significant change in E-cadherin mRNA expression (*t*-test, $P>0.05$). (E) By contrast, western blotting analysis indicated a significant increase in E-cadherin protein expression following SP600125 treatment. Scale bars: 25 µm.

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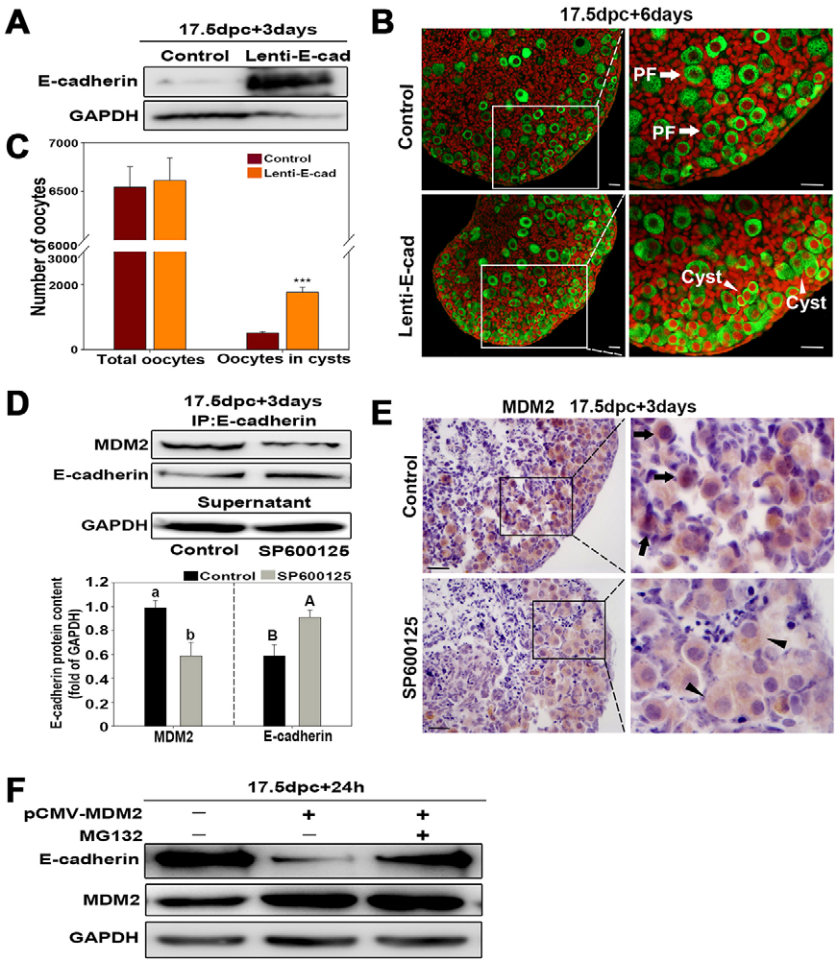

Fig. 4. JNK might regulate E-cadherin degradation through MDM2. (A) The transfection efficiency of Lenti-E-cad examined by western blotting. E-cadherin expression was clearly increased in 17.5 dpc ovaries after 3 days of culture with Lenti-E-cad. (B) 17.5 dpc ovaries were cultured with Lenti-E-cad for 6 days. Histological analysis revealed reduced cyst breakdown. (C) The total numbers of oocytes were similar between groups. Lenti-E-cad-transfected ovaries contained more oocytes within cysts than the control group. ***P<0.001 (t-test), control versus treated ovaries. (D,E) 17.5 dpc ovaries were cultured with SP600125 for 3 days *in vitro*. (D) JNK suppression resulted in a significant decrease in MDM2 but increase in E-cadherin as assessed by Co-IP analysis. Different letters within bar charts (a,b, A,B) indicate significant differences between groups (ANOVA and Holm-Sidak test). (E) Immunohistochemistry showing that MDM2 is expressed in oocytes and exhibited a weaker staining in the SP600125-treated (arrowheads) than in the control (arrows) group. (F) 17.5 dpc ovaries were transfected with the MDM2-overexpressing vector pCMV-MDM2 for 24 h, which resulted in the E-cadherin protein being markedly degraded. However, pretreatment with 5 μM MG132 (a proteasome inhibitor) blocked the E-cadherin degradation induced by overexpression of MDM2. Scale bars: 25 μm.

follicle formation, including *Kit*, kit ligand (*Kitl*), *Fst* and *Nobox*, as well as of components in the Notch pathway were analyzed by quantitative reverse transcription PCR (qRT-PCR). The results showed that the mRNA levels of *Kit*, *Kitl*, *Fst* and *Jag2* were reduced after 3 days of SP600125 treatment (Fig. S1A). Considering that JNK was localized in the oocytes of perinatal ovaries, we decided to focus on the oocyte-expressed genes *Kit* and *Fst* and not on the somatic cell-expressed genes *Kitl* and *Jag2*. However, the protein levels of KIT and FST were unchanged following SP600125 treatment as assessed by western blotting and immunohistochemistry analyses (Fig. S1B,C), indicating that JNK signaling might control cyst breakdown and primordial follicle formation through other factors.

A previous study in hamsters reported that primordial follicle formation requires the proper spatiotemporal expression and action of E-cadherin (Wang and Roy, 2010). We therefore examined the cellular localization and expression of E-cadherin during the perinatal period. E-cadherin was intensely expressed at oocyte-oocyte contact sites inside cysts in 17.5 dpc ovaries (Fig. 3A, arrows), although in some oocytes the staining appeared to be partly diffuse. In 19.5 dpc ovaries, almost all the oocytes exhibited weak and diffuse E-cadherin expression. Later, in the 2 dpp ovaries, E-cadherin expression seemed to rise again in the oocytes and, by 4 dpp, E-cadherin was highly expressed in oocytes as well as somatic cells (Fig. 3A, arrowheads). In addition, western blotting was conducted to quantitatively analyze E-cadherin expression during this period. This showed a V-shaped pattern: E-cadherin was highly expressed at 17.5 dpc, exhibited a sharp decrease at 19.5 dpc, a mild rise at 2 dpp, and then peaked at 4 dpp (Fig. 3B).

Next, to clarify the correlation between JNK signaling and E-cadherin, we cultured 17.5 dpc ovaries with SP600125 and performed immunofluorescence staining to detect E-cadherin expression. Co-staining of β-catenin, E-cadherin and the cell nucleus suggested that JNK inhibition resulted in enhanced expression of E-cadherin and its partner β-catenin at oocyte-oocyte contact sites inside cysts (Fig. 3C, arrows), whereas their expression was weak in the control group (Fig. 3C, arrowheads). Moreover, qRT-PCR and western blotting revealed that SP600125 treatment resulted in elevated protein, but not mRNA, levels of E-cadherin (Fig. 3D,E). We also analyzed other cell junction-related factors. N-cadherin and vimentin were detected in pre-granulosa cells, and SP600125 treatment had no impact on their localization and expression (Fig. S2A). The tight junction proteins ZO-1 (TJP1) and occludin were hardly detected in fetal mouse ovaries (Fig. S2B). Moreover, the polarity protein PARD6A was strongly localized in oocytes but was unaffected by JNK signaling inhibition (Fig. S2C). These data indicate that the failure in cyst breakdown induced by JNK inhibition might be caused by disruption to E-cadherin junctions.

JNK might regulate E-cadherin degradation through MDM2

To further explore the significance of E-cadherin decline during germline cyst breakdown, E-cadherin-overexpressing lentivirus (Lenti-E-cad) was generated. Ovaries at 17.5 dpc were incubated

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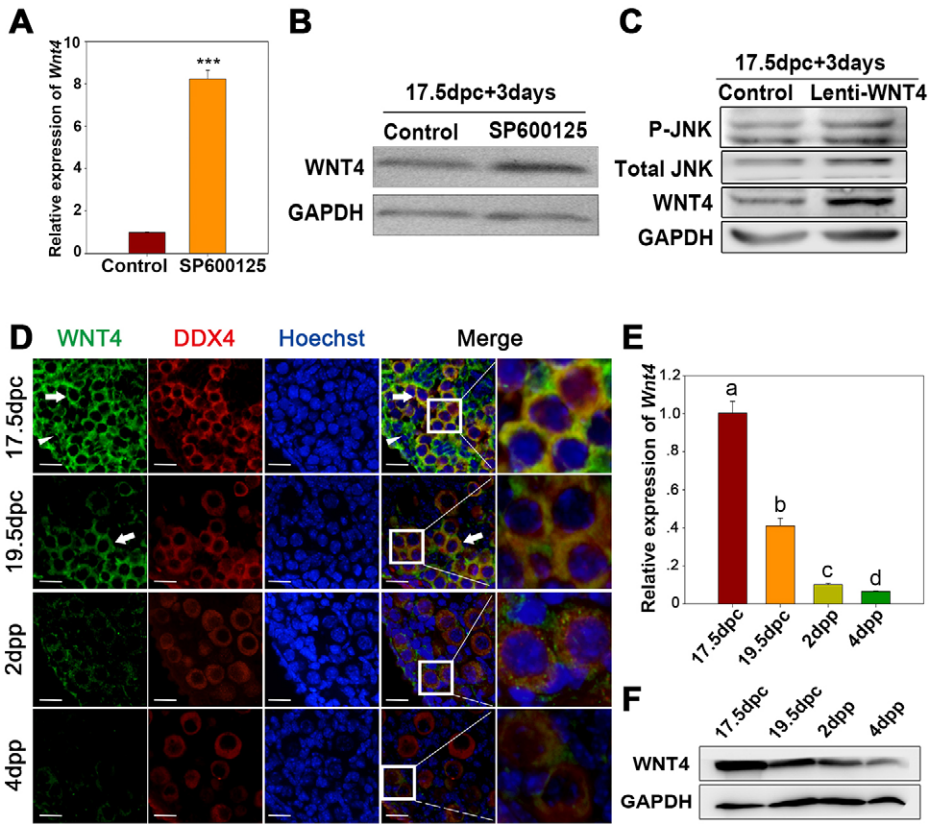

Fig. 5. JNK inhibition leads to increased expression of WNT4. (A-C) Ovaries at 17.5 dpc were treated with 5 µM SP600125 or Lenti-WNT4 for 3 days *in vitro*. (A) qRT-PCR analysis indicated a significant increase in *Wnt4* mRNA expression (*t*-test, ***P<0.001). (B) Western blotting analysis indicated a significant increase in WNT4 protein expression following SP600125 treatment. (C) JNK signaling was unaffected by WNT4 overexpression, as assessed by western blotting. (D) Cellular localization of WNT4 in the perinatal ovaries. Ovaries were stained for WNT4 and DDX4 at 17.5 dpc, 19.5 dpc, 2 dpp and 4 dpp. Hoechst was used to identify nuclear DNA. WNT4 was highly expressed in both oocytes and somatic cells at 17.5 dpc (arrow), but was only expressed in the oocytes at 19.5 dpc (arrow). After birth, the oocytes in the 2 dpp ovaries exhibited weak staining of WNT4, and WNT4 could not be detected in 4 dpp ovaries. Scale bars: 25 µm. (E) qRT-PCR analysis of *Wnt4* expression in the ovaries from 17.5 dpc to 4 dpp. *Wnt4* mRNA levels decreased with ovarian development. Different letters (a-d) indicate significant differences between groups (ANOVA and Holm-Sidak test). (F) The decreasing *Wnt4* mRNA expression observed in mouse fetal ovaries from 17.5 dpc to 4 dpp was confirmed by WNT4 protein levels, as assessed by western blot.

with Lenti-E-cad or empty lentivirus (Lenti-pLVX-IRES-ZsGreen1, as a control) for 3 days, and western blotting showed a significant increase in E-cadherin in the Lenti-E-cad group (Fig. 4A). Next, ovarian histology was evaluated after 6 days of culture. The control ovaries were chiefly composed of primordial follicles (Fig. 4B, arrows), whereas cyst breakdown was obviously delayed in Lenti-E-cad-infected ovaries (arrowheads). These results imply that E-cadherin junctions between oocytes are required to maintain cyst structure and that their disassembly initiates cyst breakdown.

Since E-cadherin protein but not mRNA levels were regulated by JNK signaling, we examined the expression of MDM2, a RING finger-containing E3 enzyme that has been reported to regulate E-cadherin protein levels (Adhikary et al., 2014; Yang et al., 2006), in the control and JNK-inhibited ovaries. Ovaries at 17.5 dpc were cultured with SP600125 for 3 days and then harvested for a co-immunoprecipitation experiment. As shown in Fig. 4D, endogenous MDM2 binds to E-cadherin during germline cyst breakdown. SP600125 treatment resulted in significantly decreased interaction of MDM2 with E-cadherin. In addition, MDM2 localization was assessed by immunohistochemistry. The results showed that MDM2 is expressed in oocyte cytoplasm (Fig. 4E, arrows) and that its staining is weaker in SP600125-treated than in control ovaries (Fig. 4E, arrowheads).

To further examine the interaction between MDM2 and E-cadherin, 17.5 dpc ovaries were cultured and transfected with MDM2-overexpressing vector (pCMV-MDM2) for 24 h and E-cadherin expression detected by western blotting. The results revealed that MDM2 overexpression markedly reduced E-cadherin expression, whereas treatment with MG132, a proteasome inhibitor, blocked MDM2-mediated E-cadherin degradation (Fig. 4F). These results imply that JNK signaling might be involved in inducing MDM2 expression, which then mediates E-cadherin protein degradation during germline cyst breakdown.

JNK inhibition leads to increased WNT4 expression

It has been widely reported that E-cadherin expression is crucial for cell morphogenesis, and that E-cadherin loss is a key initial step in the epithelial-mesenchymal transition (EMT) process (Heuberger and Birchmeier, 2010). Considering the downtrend in the pattern of E-cadherin expression during perinatal ovary development, we assumed cyst breakdown to be an EMT-like process. To verify this idea, the expression of WNT4, which is necessary for maintenance of the epithelial phenotype and regulates the EMT process in multiple cell types (Boyle et al., 2011; Carroll et al., 2005; Wang et al., 2013), was examined in SP600125-treated ovaries. qRT-PCR and immunoblotting showed that WNT4 mRNA and protein levels were markedly increased following 3-day SP600125 treatment (Fig. 5A,B). When P-JNK and total JNK levels were examined in WNT4-overexpressing ovaries, the results indicated that WNT4 overexpression had no effect on JNK signaling (Fig. 5C).

Based on the above results, we examined the WNT4 expression pattern *in vivo* when cyst breakdown occurs. Immunofluorescence was applied to firmly establish the cellular localization of WNT4 protein during perinatal ovarian development, and the results revealed that WNT4 was highly expressed in both oocytes (Fig. 5D, arrow) and somatic cells (arrowhead) at 17.5 dpc, but confined to within oocytes at 19.5 dpc (arrow). After birth, the oocytes in the 2 dpp ovaries exhibited weak WNT4 staining, and by 4 dpp WNT4 was undetectable (Fig. 5D). In addition, the WNT4 expression pattern was analyzed by qRT-PCR. As shown in Fig. 5E, *Wnt4* mRNA is highly expressed in 17.5 dpc and 19.5 dpc ovaries but decreases significantly after birth. The mRNA results were confirmed by measuring WNT4 protein levels via western

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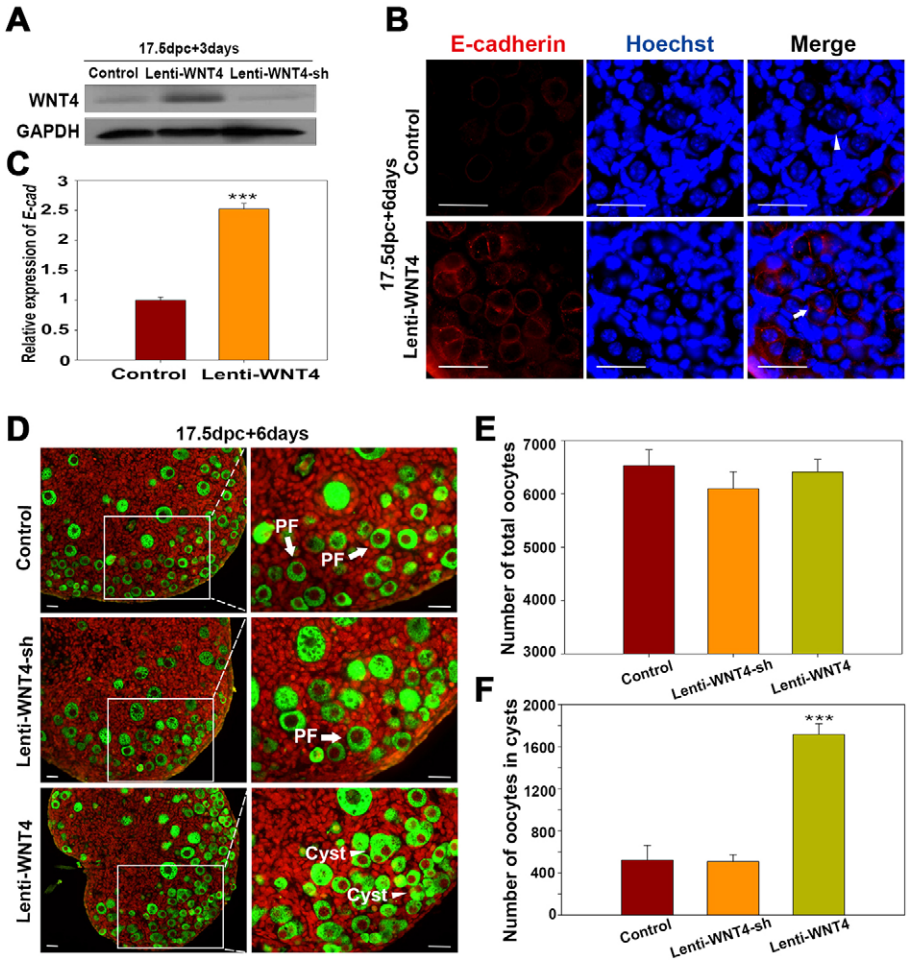

Fig. 6. WNT4 decline is required for normal germline cyst breakdown. Ovaries at 17.5 dpc were cultured with lentiviruses for 3 days or 6 days *in vitro*. (A) Western blot was performed to evaluate the efficiency of Lenti-WNT4-sh and Lenti-WNT4. WNT4 expression was clearly increased or decreased following transfection with Lenti-WNT4 or Lenti-WNT4-sh for 3 days, respectively. (B) Immunofluorescence was performed to examine E-cadherin expression after Lenti-WNT4 treatment for 6 days. WNT4 overexpression enhanced the localization of E-cadherin at oocytes inside cysts (arrow), whereas E-cadherin expression was weak in the control group (arrowhead). (C) qRT-PCR analysis of E-cadherin expression following Lenti-WNT4 treatment for 6 days. The overexpression of WNT4 induced an increase in E-cadherin mRNA expression. (D) 17.5 dpc ovaries were cultured with Lenti-WNT4-sh or Lenti-WNT4 for 6 days. Histological analysis showed that many oocytes remained within cysts in Lenti-WNT4-transfected ovaries. (E,F) Total numbers of oocytes were similar between groups. Lenti-WNT4-transfected ovaries contained more oocytes within cysts than the control group. ***P<0.001 (t-test), control versus treated ovaries. Scale bars: 25 µm.

blotting analysis (Fig. 5F). Taken together, the downtrend in the WNT4 expression pattern indicates that it is mainly expressed in the oocytes inside germline cysts prior to primordial follicle formation.

WNT4 decline is required for normal germline cyst breakdown

To better understand the role of WNT4 in cyst breakdown and primordial follicle formation, WNT4-overexpressing lentivirus (Lenti-WNT4) and WNT4 knockdown lentivirus (Lenti-WNT4-sh) were generated. When ovaries at 17.5 dpc were incubated with empty lentivirus (as a control), Lenti-WNT4 or Lenti-WNT4-sh for 3 days, western blotting showed that Lenti-WNT4 or Lenti-WNT4-sh infection could induce a significant WNT4 increase or decrease, respectively (Fig. 6A). Resembling SP600125-treated ovaries, immunofluorescent staining showed pronounced E-cadherin expression at the oocyte-oocyte contact sites in ovaries transfected with Lenti-WNT4 for 6 days, whereas E-cadherin expression was almost undetectable in the control group (Fig. 6B). qRT-PCR experiments also revealed an obvious increase in E-cadherin mRNA levels in Lenti-WNT4-transfected ovaries (Fig. 6C).

Ovaries at 17.5 dpc were incubated with empty lentivirus, Lenti-WNT4-sh or Lenti-WNT4 for 6 days and their histology evaluated. The germline cysts in control ovaries and Lenti-WNT4-sh-infected ovaries underwent programmed breakdown to form primordial follicles (Fig. 6D, arrows). By contrast, cyst breakdown was delayed in the Lenti-WNT4 group (Fig. 6D, arrowheads). Quantification of oocytes revealed that significantly more oocytes remained within cysts in Lenti-WNT4-transfected ovaries compared with the control and Lenti-WNT4-sh groups (Fig. 6E,F). These results imply that WNT4 might negatively regulate germline cyst breakdown and primordial follicle formation.

WNT4 and JNK signaling collaboratively regulate primordial follicle formation

Since WNT4 expression was elevated following JNK inhibition and correlated with E-cadherin junctions inside cysts, we investigated how JNK and WNT4 interact to drive cyst breakdown. Ovaries at 17.5 dpc were cultured with no treatment (as a control) or with 5 µM SP600125 plus Lenti-WNT4-sh or plus Lenti-WNT4. After 6 days of culture, the ovaries were prepared for sectioning and histological evaluations. The results showed that there was no significant rescue upon WNT4 knockdown of the cyst breakdown failure induced by JNK inhibition (Fig. 7A,C). Nevertheless, few primordial follicles existed in ovaries treated with SP600125 plus Lenti-WNT4, representing a more serious cyst breakdown failure than that of the SP600125 group (Fig. 7A,C). Moreover, oocyte quantification demonstrated that ovaries treated with SP600125 plus Lenti-WNT4 exhibited fewer total oocytes than the control group (Fig. 7B). Thus, programmed cyst breakdown and primordial follicle formation require simultaneous WNT4 downregulation and JNK upregulation.

DISCUSSION

The reproductive lifespan of mammalian females is determined primarily by the establishment of a pool of primordial follicles. Our

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study reveals that JNK signaling participates in the regulation of primordial follicle formation, probably by acting on the E-cadherin junctions during the cyst period. In addition, WNT4 levels within oocytes are closely correlated with this process.

Cell-cell adhesion is crucial for various aspects of multicellular existence, including morphogenesis, tissue integrity and differentiation (Gumbiner, 1996). E-cadherin-mediated adhesion acts as a modulator of PGC development (Di Carlo and De Felici, 2000). Here we demonstrated that E-cadherin is expressed intensely during the cyst period, whereas its expression decreases when cyst breakdown occurs, which is in accordance with similar findings reported in hamster (Wang and Roy, 2010) and human (Smith et al., 2010) fetal ovaries. The research carried out in hamsters also demonstrated that blocking E-cadherin action accelerates cyst breakdown and primordial follicle formation, consistent with our results that E-cadherin overexpression in mouse fetal ovaries substantially suppresses cyst breakdown. Together, these data suggest that the gradual loss of E-cadherin expression or function in oocytes is essential for the germline cyst breakdown that enables the regular release of individual oocytes to form primordial follicles.

During morphogenesis, intercellular adhesive contacts must be flexible enough to permit rearrangement. We demonstrated here that JNK signaling inhibition clearly affects the rearrangement of E-cadherin junctions during perinatal ovary development. The novel function of JNK in this process is consistent with its general role in regulating communication and cohesion between neighboring cells. For example, the rapid adherens junction disassembly of T84 and SK-CO15 cell monolayers was accompanied by JNK activation and prevented by JNK inhibition (Naydenov et al., 2009), similar to our observations in JNK-inhibited fetal ovaries. Here too, as we observed in the SP600125-treated ovaries, E-cadherin and β-catenin are upregulated when JNK function is disrupted (Lee et al., 2009; You et al., 2013). Furthermore, JNK activation has been associated with E-cadherin inactivation and cell polarity loss in *Drosophila* epithelial cells (Igaki et al., 2006). Thus, the role of JNK signaling in regulating oocyte adhesion during germline cyst breakdown is consistent with its known functions in a diverse array of morphogenetic events.

The role of WNT4 in female sexual differentiation has been extensively studied over the past 20 years (Kim et al., 2006; Vainio et al., 1999). Generally accepted as a female determining gene, *Wnt4* is highly expressed to promote ovarian differentiation but downregulated in male gonads after the initiation of testicular formation at 11.5 dpc (Vainio et al., 1999). However, it was not clear whether WNT4 is continuously expressed until the primordial follicle formation period (17.5 dpc to 4 dpp) and, if so, what its function might be. Here we showed that WNT4 is constantly expressed in fetal ovaries until primordial follicle formation. It is particularly expressed in oocytes during the germline cyst period, yet its expression is instantly decreased after birth. Importantly, we provide evidence that WNT4 impacts E-cadherin junctions between oocytes inside cysts, as well as being involved in cyst structure maintenance.

Our results revealed a novel role of JNK that involves the inhibition of WNT4 expression, which corresponds with previous findings in which JNK1-deficient embryonic stem cells showed inhibited

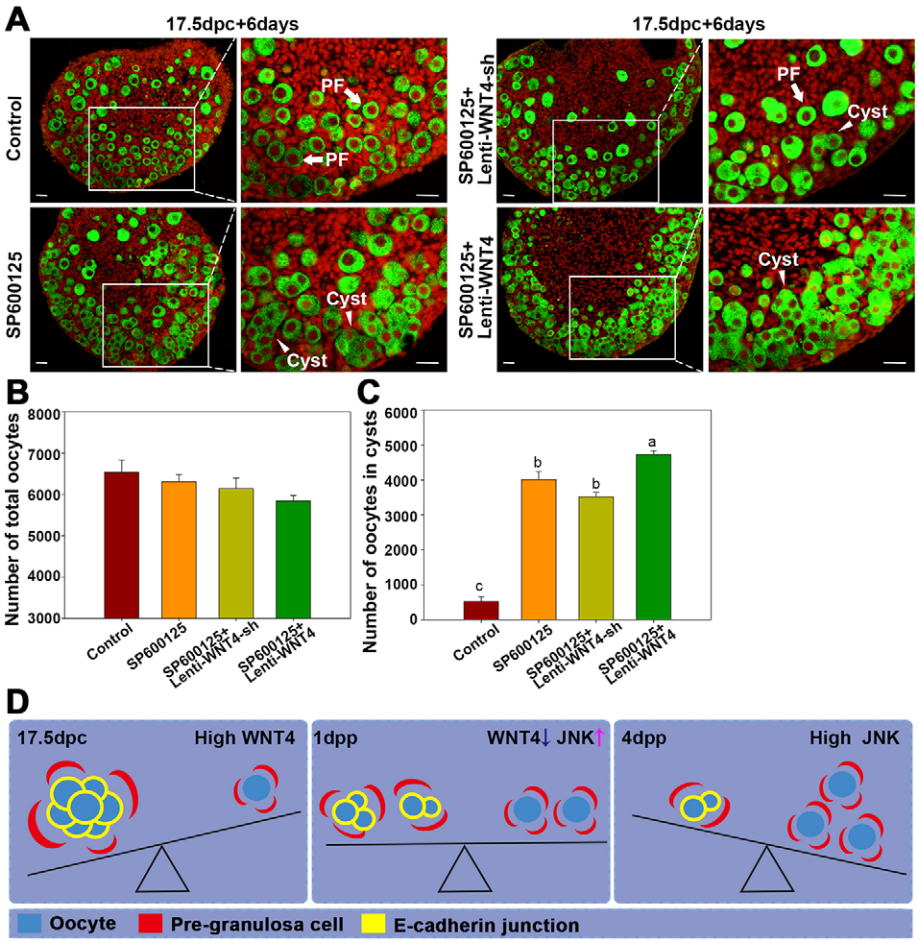

Fig. 7. WNT4 and JNK signaling coordinate germline cyst breakdown. (A) Ovaries at 17.5 dpc were cultured with SP600125, SP600125 plus Lenti-WNT4-sh, or SP600125 plus Lenti-WNT4. After 6 days of culture, sections of ovary were immunolabeled for DDX4 (green) and stained with PI (red). Lenti-WNT4-sh treatment could not significantly rescue the SP600125-induced cyst breakdown failure, but Lenti-WNT4 treatment enhanced the severity of the SP600125-induced phenotype. Scale bars: 25 µm. (B,C) Total numbers of oocytes were similar between groups. There was no significant change in the number of oocytes within cysts between SP600125 and SP600125 plus Lenti-WNT4-sh treatment. SP600125 plus Lenti-WNT4-transfected ovaries had even more oocytes within cysts than the SP600125 group. Different letters (a-c) indicate significant differences between groups (ANOVA and Holm-Sidak test). (D) Model of JNK and WNT4 signaling function in cyst breakdown and primordial follicle formation. E-cadherin junction-dependent oocyte adhesion was sustained by high expression of WNT4 during the cyst period. Then, JNK signaling showed an uptrend, while the level of WNT4 decreased simultaneously. This reciprocal change leads to the disassembly of E-cadherin junctions and the gradual breakdown of germline cysts. When the JNK signaling predominates at 4 dpp, oocytes are completely released from cysts and surrounded by pre-granulosa cells to form primordial follicles.

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neurogenesis associated with increased WNT4 and E-cadherin expression (Amura et al., 2005). In addition, JNK signaling antagonizes the canonical WNT3A pathway by regulating β-catenin transport (Liao et al., 2006). Our data show that JNK signaling attenuation leads to WNT4 upregulation, but knockdown of WNT4 could not significantly rescue the JNK inhibition-induced cyst breakdown failure. In addition, WNT4 regulates E-cadherin mRNA levels, whereas JNK signaling does not. Based on these results, we hypothesize that WNT4 controls E-cadherin transcription during the early cyst period, but that JNK signaling is required for E-cadherin degradation and oocyte adhesion disassembly when cyst breakdown occurs. Taken together, these two signaling pathways might regulate E-cadherin junctions through different mechanisms during germline cyst breakdown.

Our previous study showed that a strictly programmed oocyte-somatic cell interaction is essential for normal folliculogenesis (Lei et al., 2006). Existing studies have shown that somatic cells participate in cyst breakdown and primordial follicle formation (Trombly et al., 2009; Xu and Gridley, 2013) but studies specific to oocyte characteristics are lacking. Our recent study demonstrated that cyclic AMP (cAMP) in oocytes regulates oocyte meiotic prophase I and primordial folliculogenesis (Wang et al., 2014). Here, we proved that JNK and WNT4 signaling in oocytes function coordinately on oocyte adhesion to regulate cyst breakdown and primordial follicle formation. In summary, the collaboration between oocytes and somatic cells is indispensable for normal folliculogenesis.

Based on the results presented here, we propose a model of how germline cyst breakdown is controlled in mice (Fig. 7D). E-cadherin junction-dependent oocyte adhesion is sustained by high WNT4 expression during the cyst period. JNK signaling activity shows an uptrend, while the WNT4 level decreases simultaneously. This reciprocal change leads to E-cadherin junction disassembly and gradual germline cyst breakdown. JNK signaling predominates at 4 dpp, at which point the oocytes are completely released from the cysts and surrounded by pre-granulosa cells to form the primordial follicles. In summary, a dynamic balance between JNK and WNT4 activity is essential for cyst structure maintenance and breakdown during the early stage of ovarian development.

Our study provides a new framework for studying the mechanism of primordial follicle formation and has broad physiological and clinical implications for increasing our understanding of ovarian pathology.

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 (6-8 weeks old) were mated with males overnight and those with a vaginal plug were considered as 0.5 dpc. Mice were maintained with free access to food and water under a 16/8 h light/dark cycle. All animal experiments were performed in accordance with the guidelines and regulations of the Institutional Animal Care and Use Committee of China Agricultural University.

Ovary isolation and culture

Ovaries were dissected carefully from the mesonephros as described previously (Wen et al., 2009) and then cultured in 1 ml DMEM/F12 medium (GIBCO, Life Technologies) at 37°C in an atmosphere of 5% CO_2. The medium was supplemented with streptomycin (50 µg/ml, Sigma-Aldrich) and penicillin (60 µg/ml, Sigma-Aldrich) to prevent bacterial contamination. To assess the function of JNK signaling in primordial follicle formation, cultured ovaries were treated with 5 µM SP600125 (S5567, Sigma-Aldrich), which is a selective JNK signaling pathway inhibitor (Bennett et al., 2001). The proteasome inhibitor MG132 was obtained from Dr Haibin Wang (Institute of Zoology, Chinese Academy of Sciences). The final concentration of DMSO in any solution used throughout the study did not exceed 0.1%.

Lentivirus production and ovary infection

Lentiviruses were produced in HEK 293T cells by cotransfecting 5 µg pMD2.G, 15 µg psPAX2 and 20 µg transfer vector (pSicoR or pLVX-IRES-ZsGreen1). JNK1/2-shRNA and WNT4-shRNA lentiviruses were constructed by cloning JNK or WNT4 shRNAs (Table S1) into the pSicoR vector. E-cadherin-overexpressing and WNT4-overexpressing lentiviruses were constructed by cloning the open reading frame of E-cadherin or WNT4 into the pLVX-IRES-ZsGreen1 vector. The transfection was performed with Lipofectamine 3000 (Invitrogen) and the transfection medium was replaced 6 h post-transfection. The viral supernatants were harvested at 24 and 48 h using a 0.45 µm membrane and centrifuged at 57,400 ***g*** at 4°C for 2 h. The lentiviruses were injected into the ovary using a thin glass needle with a mouthpiece. For each injection, the optimal volume was 0.3 µl per ovary. The lentiviral constructs pMD2.G and psPAX2 were obtained from Dr Sheng Cui (China Agricultural University) and pSicoR and pLVX-IREX-ZsGreen1 were obtained from Dr Haibin Wang.

Histological sections and oocyte counts

Collected ovaries were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and sectioned to a thickness of 5 µm. The sections were stained with DDX4 antibody and every fifth section was analyzed for the presence of oocytes. A follicle containing an oocyte surrounded by a single layer of pre-granulosa cells with flattened nuclei was defined as a primordial follicle, and a structure containing clusters of oocytes connected together and with shared chaotic cytoplasm was defined as a germline cyst. The cumulative oocyte counts were multiplied by five (Flaws et al., 2001).

Immunofluorescence and immunohistochemistry

Ovaries were fixed in 4% PFA overnight, embedded in paraffin, and sectioned at 5 µm. After dewaxing, rehydration, and high-temperature (92°C) antigen retrieval with 0.01% sodium citrate buffer (pH 6.0), the sections were blocked with 10% normal serum and immunostained with primary antibodies overnight at 4°C. The anti-ZO-1, anti-occludin and anti-vimentin antibodies were a generous gift from Dr Haibin Wang. The other primary antibodies used are presented in Table S2. For immunofluorescence, the slides were then incubated with Alexa Fluor 488- or 555-conjugated secondary antibodies (1:100; Invitrogen) at 37°C for 1 h. All sections were viewed directly using a fluorescence microscope (80i, Nikon). For immunohistochemistry, the slides were incubated with biotinylated secondary antibody (Zhongshan Golden Bridge, Beijing, China) and avidin-biotin-peroxidase (Zhongshan Golden Bridge) before being exposed to diaminobenzidine (DAB; Zhongshan Golden Bridge) for 1 min and then counterstained with Hematoxylin.

Western blotting

Total protein from ovaries was extracted in western blot immunoprecipitation cell lysis buffer (WIP; CellChip Beijing Biotechnology, Beijing, China), according to the manufacturer's protocol; protein concentrations were measured by a BCA assay (CellChip Beijing Biotechnology). Proteins were separated on a 10% SDS-PAGE gel and then transferred onto polyvinylidene fluoride (PVDF) membranes (IPVH00010, Millipore). Membranes were incubated overnight at 4°C with the appropriate primary antibody (Table S2). After rinsing thoroughly with TBST (ZSGB-BIO, Beijing, China), the membranes were incubated for 1 h at room temperature with the appropriate secondary antibody (ZSGB-BIO, Beijing, China) diluted 1:5000 in TBST. Finally, the membranes were visualized using the Super Signal chemiluminescent detection system (34080, Thermo). Levels of GAPDH were used as an internal control.

Co-immunoprecipitation (Co-IP)

Co-IP protein lysates (1 mg) from culture ovaries were extracted using an immunoprecipitation kit (10007D, Novex, Life Technologies). Antibodies

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to E-cadherin, MDM2 and GAPDH (Table S2) were used. A magnet (Novex, Life Technologies) was used to separate the beads and supernatant. The Dynabeads-antibody-antigen complex was incubated with rotation for 90 min at room temperature. After complete elution and SDS denaturation, the samples were separated by SDS-PAGE and detected with appropriate antibodies.

RNA extraction and qRT-PCR

Total RNA of mouse fetal ovaries was extracted using TRIzol (Invitrogen, Life Technologies), according to the manufacturer's protocol. First-strand cDNA was generated using reverse transcription (Reverse Transcription System, Promega) from 1 µg total RNA. qPCR was performed using a QuantiTect SYBR Green PCR Kit (Qiagen) on an ABI 7500 qRT-PCR system (Applied Biosystems). Gene expression changes were analyzed by the $2^{-\Delta\Delta Ct}$ method as reported previously (Livak and Schmittgen, 2001) and normalized to *Gapdh*. The primers used are presented in Table S3.

Statistical analyses

All experiments were repeated at least three times. Data are presented as mean±s.e.m. Five ovaries per group were used for oocytes 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-Sidak test. Data were considered statistically significant at $P<0.05$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

W.N. and G.X. designed the work with input from other authors. W.N. and Ye W. performed the experiments. W.N., Z.W., Q.X., Yijing W., L.F., L.Z. and J.W. analyzed the data and contributed to reagents, materials or analysis tools. The manuscript was written by W.N. and revised by H.Z., C.W. and G.X.

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Supplementary information

Supplementary information available online at
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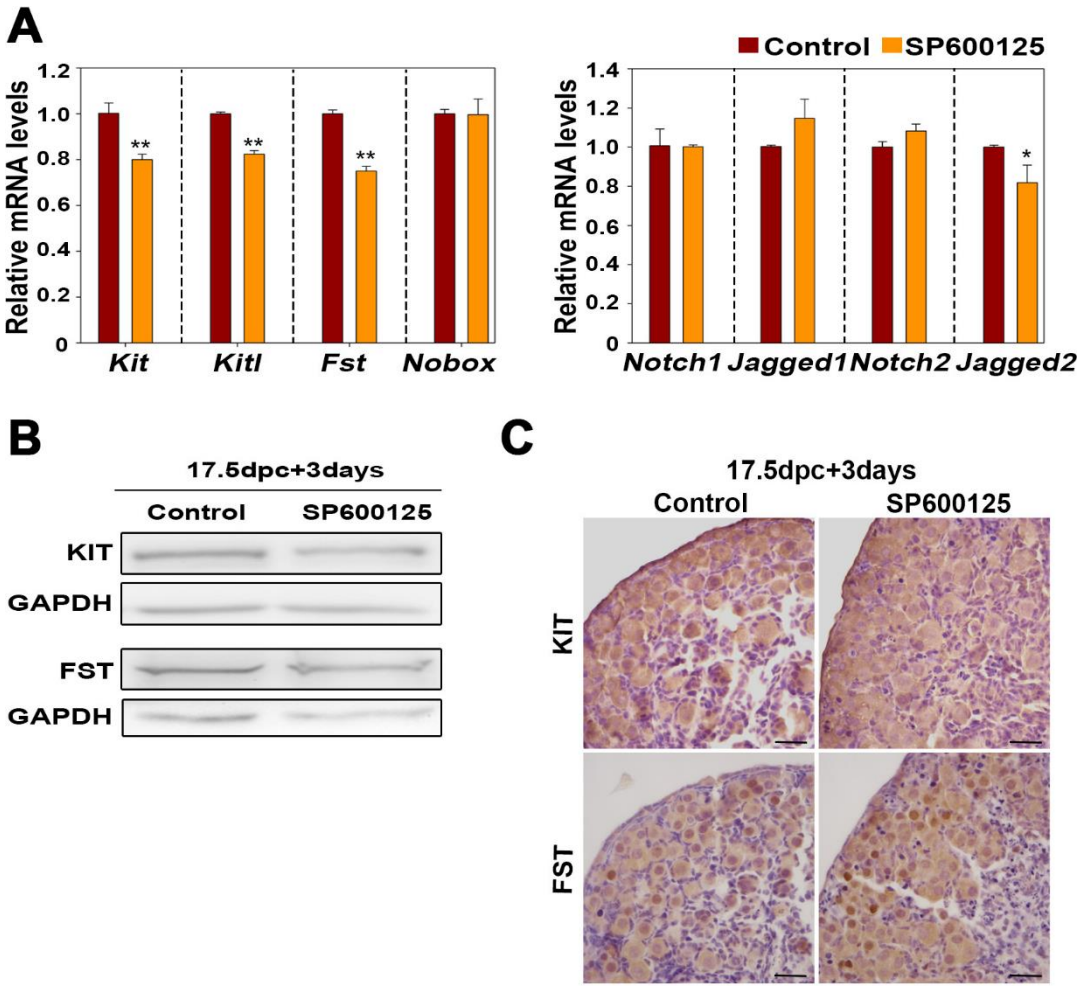

Figure S1. JNK signaling inhibition does not affect KIT, FST protein expression. 17.5 dpc ovaries were cultured with SP600125 for 3 days *in vitro*. **(A)** The mRNA levels of several primordial folliculogenesis related genes were detected using qRT-PCR. The expression of *Kit*, *Kitl*, *Fst*, and *Jagged2* were decreased following the SP600125 treatment, while the expression of *Nobox* and other *Notch* family genes exhibited mild alterations. Asterisk (*) indicates a significant difference between control and treated ovaries. $*P < 0.05$, $**P < 0.01$(*t*-test), control versus treated ovaries. **(B)** Western blotting analyses indicated no remarkable change in KIT and FST protein expression following SP600125 treatment. **(C)** KIT and FST were both mainly expressed in oocytes and not affected by JNK inhibition after immunohistochemistry analyses. Scale bars: 25 μm.

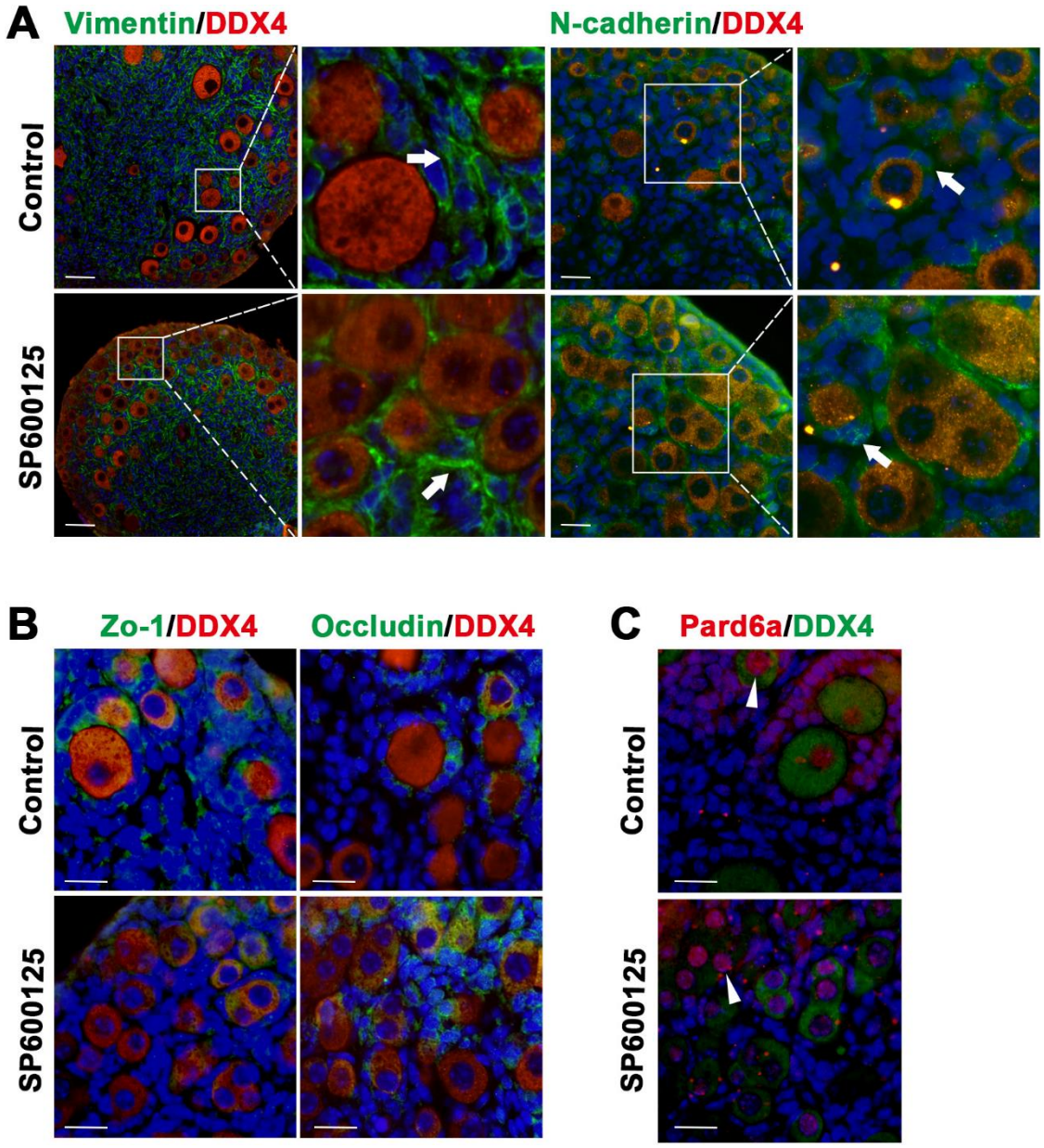

Figure S2. JNK inhibition signaling does not affect other cell junction related factors. 17.5 dpc ovaries were cultured with SP600125 for 6 days *in vitro* and immunofluorescence was performed to examine the expression of some cell junction related factors. Sections of ovary were stained with DDX4 antibody to identify oocytes. **(A)** N-cadherin (green) and Vimentin (green) were detected in pre-granulosa cells (arrows) and the SP600125 treatment had no impact on their localization and expression. **(B)** The tight junction protein Zo-1 (green) and Occludin (green) were hardly detected in fetal mouse ovaries. **(C)** The polarity protein Pard-6a (red) was strongly localized in oocytes (arrowheads), but was not affected by inhibition of JNK signaling. Scale bars: 25 μm.

Table S1. Oligonucleotide primers used for shRNA lentivirus production

Primers	Primer sequence (5'……3')	Length (bp)
JNK1-sh1-F	TGGTGCATTATGGGAGAAATTTCAAGAGAATTTCTCCC ATAATGCACCTTTTTTC	55
JNK1-sh1-R	TCGAGAAAAAAGGTGCATTATGGGAGAAATTCTCTTGA AATTTCTCCCATAATGCACCA	59
JNK1-sh2-F	TGTCCTGAATTCATGAAGAATTCAAGAGATTCTTCATGA ATTCAGGACTTTTTTC	55
JNK1-sh2-R	TCGAGAAAAAAGTCCTGAATTCATGAAGAATCTCTTGA ATTCTTCATGAATTCAGGACA	59
JNK2-sh1-F	TGGTGCATCATGGCAGAAATTTCAAGAGAATTTCTGCC ATGATGCACCTTTTTTC	55
JNK2-sh1-R	TCGAGAAAAAAGGTGCATCATGGCAGAAATTCTCTTGA AATTTCTGCCATGATGCACCA	59
JNK2-sh2-F	TGCCAACTGTAAGGAATTATTTCAAGAGAATAATTCCTT ACAGTTGGCTTTTTTC	55
JNK2-sh2-R	TCGAGAAAAAAGCCAACTGTAAGGAATTATTCTCTTGA AATAATTCCTTACAGTTGGCA	59
WNT4-sh-F	TGAGAAACTCAAAGGCCTGATTCAAGAGATCAGGCCT TTGAGTTTCTCTTTTTTC	55
WNT4-sh-R	TCGAGAAAAAAGAGAAACTCAAAGGCCTGATCTCTTG AATCAGGCCTTTGAGTTTCTCA	59
Scramble-sh-F	TGAACTCAAGACCGATATTATTCAAGAGATAATATCGGT CTTGAGTTCTTTTTTC	55
Scramble-sh-R	TCGAGAAAAAAGAACTCAAGACCGATATTATCTCTTGA ATAATATCGGTCTTGAGTTCA	59

Table S2. Primary antibodies used in the immunodetection

Antibody	Catalog Code	Source	Host	Dilution	
				IF/IHC	WB
JNK	Sc-1648	Santa Cruz	Mouse	1:100	1:500
DDX4	Ab27591	Abcam	Mouse		1:200
P-JNK	Sc-135642	Santa Cruz	Rabbit		1:500
GAPDH	AM4300	Life Technologies	Mouse		1:500
C-Jun	9165	Cell Signaling Technology	Rabbit		1:500
P-c-Jun (ser 63)	9261s	Cell Signaling Technology	Rabbit		1:200
E-cadherin	Ab76055	Abcam	Mouse	1:200	1:500
N-cadherin	Ab12221	Abcam	Rabbit	1:200	
β-catenin	Sc-7199	Santa Cruz	Rabbit	1:500	
WNT4	Ab91226	Abcam	Rabbit	1:100	1:500
MDM2	Ab178938	Abcam	Rabbit	1:200	1:500
FST	Sc-30194	Santa Cruz	Rabbit	1:200	1:200
KIT	Sc-168	Santa Cruz	Rabbit	1:200	1:200

Table S3. Primers used in qRT-PCR

Gene	Forward (5'……3')	Reverse (5'……3')
Kit	GGCCTCACGAGTTCTATTTACG	GGGGAGAGATTTCCCATCACAC
Kitl	GAATCTCCGAAGAGGCCAGAA	GCTGCAACAGGGGGTAACAT
Fst	TGCTGCTACTCTGCCAGTTC	GTGCTGCAACACTCTTCCTTG
Nobox	AAGACCCGAACCCTGTACC	CTCATGGCGTTTGTCACTGTC
Notch1	GATGGCCTCAATGGGTACAAG	TCGTTGTTGTTGATGTCACAGT
Jag1	CCTCGGGTCAGTTTGAGCTG	CCTTGAGGCACACTTTGAAGTA
Notch2	ATGTGGACGAGTGTCTGTTGC	GGAAGCATAGGCACAGTCATC
Jag2	CAATGACACCACTCCAGATGAG	GGCCAAAGAAGTCGTTGCG
Wnt4	CGGGGAAGGCCGATAATTTAAAC	CAAGGGACCCAAAAACCAAACC
E-cadherin	CAGCCTTCTTTTCGGAAGACT	GGTAGACAGCTCCCTATGACTG
Jnk1	ATGGCTGTCGATATTCAACCAG	CCTCTTGGGCATACCCCAC
Jnk2	GGGCTCCAGAAGTCATCCTG	AACCTTTCACCAGCTCTCCC