Article

SP1 governs primordial folliculogenesis by regulating pregranulosa cell development in mice

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Establishment of the primordial follicle (PF) pool is pivotal for the female reproductive lifespan; however, the mechanism of primordial folliculogenesis is poorly understood. Here, the transcription factor SP1 was shown to be essential for PF formation in mice. Our results showed that SP1 is present in both oocytes and somatic cells during PF formation in the ovary. Knockdown of *Sp1* expression, especially in pregranulosa cells, significantly suppressed nest breakdown, oocyte apoptosis, and PF formation, suggesting that SP1 expressed by somatic cells functions in the process of primordial folliculogenesis. We further demonstrated that SP1 governs the recruitment and maintenance of Forkhead box L2-positive (FOXL2^{+}) pregranulosa cells using an *Lgr5*-EGFP-IRES-*Cre*ER[T2] (*Lgr5-KI*) reporter mouse model and a FOXL2^{+} cell-specific knockdown model. At the molecular level, SP1 functioned mainly through manipulation of NOTCH2 expression by binding directly to the promoter of the *Notch2* gene. Finally, consistent with the critical role of granulosa cells in follicle survival *in vitro*, massive loss of oocytes in *Sp1* knockdown ovaries was evidenced before puberty after the ovaries were transplanted under the renal capsules. Conclusively, our results reveal that SP1 controls the establishment of the ovarian reserve by regulating pregranulosa cell development in the mammalian ovary.

Keywords: SP1, pregranulosa cells, primordial follicle formation, NOTCH2, premature ovarian insufficiency

Introduction

Ovarian follicles are the fundamental functional reproductive units in which oocytes develop in mammals (Edson et al., 2009; Li and Albertini, 2013). The formation of primordial follicles (PFs) represents the first stage of folliculogenesis and is the foundation for further development. *In vivo*, with the initiation of ovarian follicle development, the population of PFs is established perinatally and serves as a finite oocyte pool that is available during the female reproductive lifespan (Pepling and Spradling,

2001; Pepling, 2006). Clinically, failure of PF formation results in premature ovarian insufficiency (POI) (Rajkovic et al., 2004). Notably, methods to induce oocyte meiotic maturation and fertilization from PFs and even from *in vitro*-cultured stem cells have been developed (Eppig and O'Brien, 1996; Morohaku et al., 2016; Jung et al., 2017; Wang et al., 2017b). The related studies revealed that germ cells could develop into functional oocytes only after cooperating with fetal ovarian somatic cells (OSCs) to form PFs. Unfortunately, the lack of a profound understanding of the mutual interactions between germ cells and somatic cells has restricted the clinical utilization of large germ cell populations in ovaries. Therefore, an improved understanding of the mechanism of PF formation will provide insight to improve *in vitro* folliculogenesis techniques, will benefit explorations of the mysteries of *in vivo* follicle development, and might explain the possible clinical cause of POI.

In mice, after primordial germ cells (PGCs) migrate to the genital ridge, they proliferate via mitosis and develop into clusters

of germ cells called germline cysts or nests (Edson et al., 2009). Mouse female germ cells differentiate into oocytes after simultaneously undergoing diplotene arrest and Balbiani body (B-body) establishment (Wang et al., 2015; Lei and Spradling, 2016). Concomitantly, Forkhead box L2-positive (FOXL2$^+$) pregranulosa cells are recruited from leucine-rich repeat-containing G-protein-coupled receptor 5-positive (LGR5$^+$) follicle-supporting progenitor cells in the ovarian surface epithelium to prepare for the encirclement of oocytes and the formation of PFs (Mork et al., 2012; Feng et al., 2016). Immediately before birth, germline nests start to break down; then, the formation of PFs progresses through various processes, including pregranulosa cell extension of cytoplasmic projections between oocytes and selective oocyte apoptosis (Wang et al., 2017a; Fu et al., 2018).

During folliculogenesis, oocytes and pregranulosa cells undergo dynamic alterations in gene expression that are regulated by a set of well-coordinated transcription factors (TFs). Because these TFs are generally active in oocytes and somatic cells (Rajkovic et al., 2004; Schmidt et al., 2004; Jagarlamudi and Rajkovic, 2012), understanding the role of TFs that function specifically in folliculogenesis will contribute to a better understanding of the mechanism of oogenesis and provide rational signal transduction targets for improving the quality of oocyte maturation in the clinic. Unfortunately, only a few TFs have been reported to be important for PF formation (Jagarlamudi and Rajkovic, 2012). The absence of oocyte-derived NOBOX and FIGα and OSC-derived FOXL2 leads to follicle development arrest, ovarian insufficiency, and infertility (Soyal et al., 2000; Rajkovic et al., 2004; Schmidt et al., 2004). SP1, a specificity protein/Krüppel-like factor (Sp/KLF) family member, is responsible for binding to GC-rich boxes on the promoters of target genes (van Vliet et al., 2006; O'Connor et al., 2016). As the first characterized and one of the best studied TFs in mammals, SP1 contributes not only to the basal transcriptional activity of cells but also to the regulation of many genes associated with cell proliferation and differentiation (Emili et al., 1994). The activity and stability of SP1 are affected by several key signaling kinases, such as JNK, ERK1/2, and AKT (Beishline and Azizkhan-Clifford, 2015). In fact, as a ubiquitous TF, tissue- and development-specific functions of SP1 have been found in many systems with SP1 binding site mutation experiments (O'Connor et al., 2016). However, the role of SP1 in regulating ovarian development, especially in the process of PF formation, remains unknown.

In the current study, we investigated the functional role of SP1 in PF formation in the perinatal mouse ovary. We found that SP1 expressed by somatic cells plays an indispensable role in the progression of germline nest breakdown and PF formation in mice by regulating the recruitment and maintenance of FOXL2$^+$ pregranulosa cells, mainly through NOTCH2 signaling. Our findings provide additional evidence elucidating the importance of OSC development during folliculogenesis and thus contribute to a better understanding of the mechanisms of folliculogenesis and follicle survival.

Results

SP1 plays a regulatory role in the formation of PFs

To investigate the potential relationship between SP1 and PF formation, immunofluorescence staining and western blot assays were employed to detect the cellular localization and expression dynamics of SP1 in perinatal ovaries. SP1 was present primarily in somatic cells in 16.5 days post coitum (dpc) ovaries, and during follicle establishment; it began to be expressed in both oocytes and somatic cells from 18.5 dpc to 3 days postpartum (dpp) (Figure 1A). Accordingly, the protein levels of SP1 in ovaries increased from 16.5 dpc to 1 dpp during primordial folliculogenesis; with establishment of the PF pool at 3 dpp, the expression of SP1 decreased dramatically (Figure 1B). These results indicate that SP1 might play a regulatory role in the formation of PFs.

To determine the precise effect of SP1 on the regulation of primordial folliculogenesis, a lentivirus carrying *Sp1* shRNA (Lenti-*Sp1*-sh) with a GFP reporter was employed in cultured fetal ovaries. Cultured 15.5 dpc ovaries were infected with Lenti-*Sp1*-sh or a scrambled control (SCR-sh). After 5 days of treatment, GFP was observed throughout the entire ovary, indicating successful infection (Supplementary Figure S1A). Western blot and immunofluorescence analysis of SP1 expression showed that *Sp1* was efficiently knocked down (Supplementary Figure S1B–D). In addition, histological analysis and follicle number counts showed that after 5 days of Lenti-*Sp1*-sh treatment, the number of PFs had significantly decreased (1546 ± 74.5 per ovary for *Sp1* knockdown vs. 3763 ± 153.1 per ovary for control; Figure 1C and D). When the treated ovaries were cultured for up to 8 days (equivalent to 3 dpp *in vivo*), most oocytes (6740.0 ± 259.8 oocytes per ovary; Figure 1E) in the control group were surrounded by pregranulosa cells and formed PFs (Figure 1C, arrows). In contrast, only a few oocytes were surrounded by pregranulosa cells to form PFs in *Sp1* knockdown ovaries (2401.0 ± 629.9 per ovary; Figure 1E), and the majority of oocytes within the ovaries were retained in nests (Figure 1C, arrowheads). In addition, consistent with the extensive oocyte apoptosis that occurred during PF formation *in vivo* (Supplementary Figure S2A; Edson et al., 2009; Pepling, 2012), the number of total oocytes in the control ovaries (7275.0 ± 334.9 oocytes per ovary) after 8 days of *in vitro* culture was markedly lower than that after 5 days of culture (12188.0 ± 247.6 oocytes per ovary) (Figure 1D and E; Supplementary Figure S2B). However, in the Lenti-*Sp1*-sh-treated group, there were no obvious changes in oocyte numbers between 5 (14244.0 ± 680.5 oocytes per ovary) and 8 days of culture (12118 ± 382.5 oocytes per ovary) *in vitro* (Figure 1D and E; Supplementary Figure S2B), suggesting that *Sp1* knockdown likely prevented oocyte apoptosis. Moreover, the ability of an SP1-selective inhibitor (mithramycin A, MIT) to block PF formation, similar to *Sp1* knockdown, further confirmed the functional role of SP1 (Supplementary Figure S3A and B). Therefore, SP1 is essential for controlling the formation of PFs in the mouse ovary.
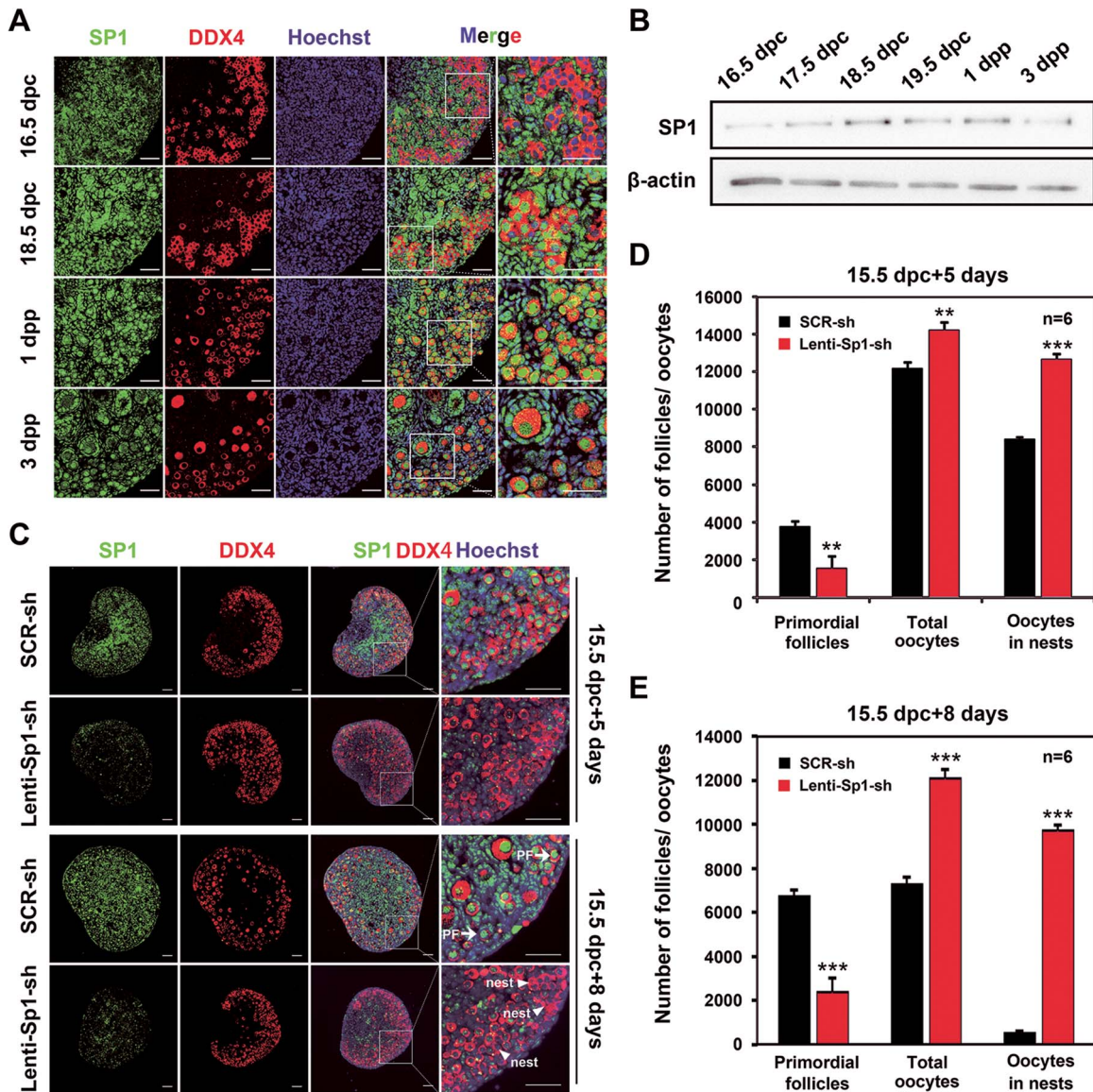
Figure 1 SP1 plays a regulatory role in the formation of PFs. (**A**) SP1 was expressed in both oocytes and somatic cells from 16.5 dpc to 3 dpp. SP1 in green; oocyte in red (DDX4); nuclear DNA in blue (Hoechst). Scale bar, 40 µm. (**B**) The protein levels of SP1 from 16.5 dpc to 3 dpp. (**C–E**) Ovaries phenotype analysis after RNAi. (**C**) The cell phenotypes and lentivirus infection efficiency were examined using an SP1 (green) immunostaining assay after 5 and 8 days of culture. Oocytes were stained for DDX4 (red), and nuclei were dyed with Hoechst (blue). PF: arrows; nest: arrowheads; scale bar, 40 µm. (**D** and **E**) Quantification of the PFs, total oocytes, and oocytes in nests per ovary after 5 (**D**) and 8 days (**E**) of culture. The data are presented as mean ± SEM. **$P < 0.01$ and ***$P < 0.001$ (t-test).

Sp1 knockdown has no obvious effect on oocyte development in perinatal ovaries

PF formation is well known to require synchronous development of oocytes and somatic cells (Lei et al., 2006). Given that SP1 was expressed in both oocytes and OSCs during primordial folliculogenesis (Figure 1A), an *in vitro* reconstitution system was applied to determine which cell type employed SP1 to accomplish PF formation (Figure 2A). The results showed that oocytes treated with the SP1 inhibitor successfully interacted with OSCs from the untreated group and transformed into PF-like

structures (Figure 2B, arrows), whereas the OSCs treated with the SP1 inhibitor were unable to surround oocytes isolated from untreated ovaries (Figure 2B, arrowheads), suggesting that SP1 likely functions in somatic cells rather than in oocytes during PF formation.

Under physiological conditions, oocytes undergo a series of critical processes that are required for PF formation, such as diplotene arrest of meiosis, organelle enrichment for oocyte differentiation, and expression changes in genes related to oocyte interactions with pregranulosa cells (Rajkovic, 2004;
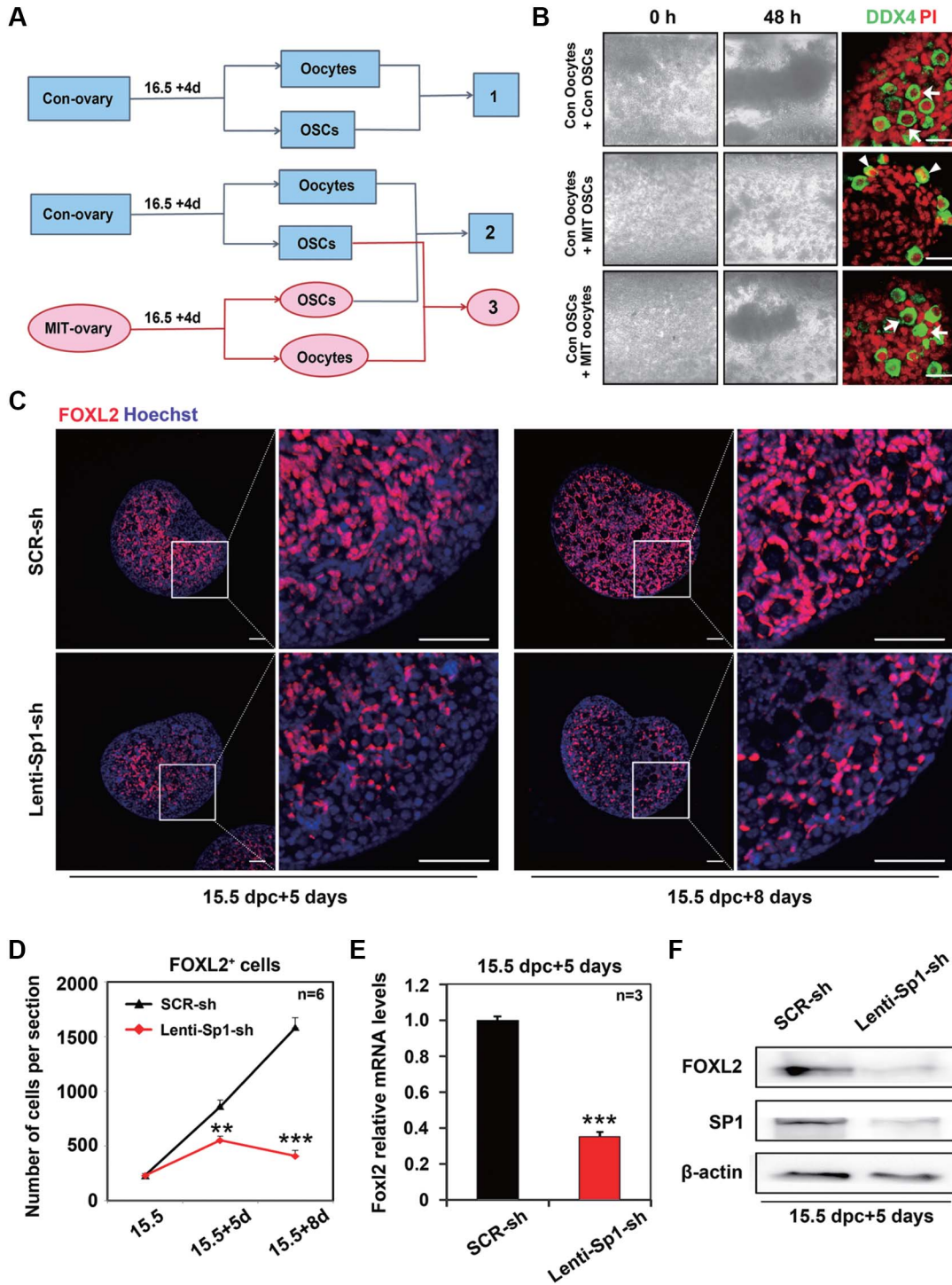
Figure 2 *Sp1* knockdown decreases the number of FOXL2$^+$ cells in perinatal ovaries. (**A** and **B**) SP1 functioned in OSCs for PF formation. (**A**) Ovaries at 16.5 dpc were cultured for 4 days, and the ovarian cells were dispersed and reconstituted into follicle-like structures (group 1). Before reconstitution, oocytes and OSCs derived from ovaries pretreated with or without MIT (groups 2 and 3) were exchanged. (**B**) The tissue structures of the reconstructed cell masses. DDX4: green; propidium iodide: red. Arrows: follicle-like structures, arrowheads: OSCs treated with MIT failed to enclose oocytes. Scale bar, 20 μm. (**C**) FOXL2 immunofluorescence (red) was assessed after *Sp1* knockdown for 5 and 8 days. Nuclei: Hoechst (blue). Scale bar, 40 μm. (**D**) Quantification of FOXL2$^+$ cells after 5 and 8 days of culture. (**E** and **F**) The expression of *Foxl2* mRNA (**E**) and protein (**F**) in *Sp1* knockdown ovaries after 5 days of culture. The data are presented as mean ± SEM. **$P < 0.01$ and ***$P < 0.001$ (*t*-test).

Wang et al., 2015; Lei and Spradling, 2016). Therefore, to exclude the effect of SP1 expressed by oocytes during PF formation, we examined the developmental status of the oocytes mentioned above in *Sp1* knockdown ovaries. Assessment of oocyte meiosis progression through analysis of chromosome spreads with synaptonemal complex 3 (SYCP3) staining and MSY2 immunostaining revealed that all the oocytes completely entered the meiotic diplotene stage in both control and *Sp1* knockdown ovaries (Supplementary Figure S4B and C); the stages of prophase I were defined by the appearance of axial elements, as previously described (Supplementary Figure S4A) (Wang et al., 2015). Moreover, normal staining of GM130 was observed in *Sp1* knockdown oocytes, suggesting that the Golgi apparatus and probably the B-body were not significantly affected in these oocytes (Supplementary Figure S4D and E). These findings indicated that *Sp1* knockdown had no effect on the organelle enrichment of oocytes. Additionally, the mRNA levels of PF formation-related genes specifically expressed in oocytes were similar between the control and Lenti-*Sp1*-sh treatment groups (Supplementary Figure S4F). Therefore, oocyte-derived SP1 probably has no effect on primordial folliculogenesis.

Sp1 knockdown decreases the number of FOXL2+ pregranulosa cells in perinatal ovaries

Previous studies have shown that FOXL2+ cells surround oocytes as pregranulosa cells and form PFs (Mork et al., 2012). Given that SP1 likely functions in somatic cells (Figure 2A and B), we examined the role of SP1 in modulating the development of FOXL2+ pregranulosa cells. After 5 days of culture, *Sp1* knockdown had significantly decreased the number of FOXL2+ cells in the knockdown group compared with the control group (549 ± 41.0 cells per section for *Sp1* knockdown vs. 859 ± 58.7 cells per section for control; Figure 2C and D). When the ovaries were cultured for up to 8 days (equivalent to 3 dpp *in vivo*), the number of FOXL2+ cells in control ovaries (1583 ± 88.9 cells per section) was markedly higher than that in ovaries cultured for 5 days (859 ± 58.7 cells per section) (Figure 2C and D). In contrast, slightly fewer FOXL2+ cells were found in *Sp1* knockdown ovaries after 8 days of culture (406 ± 52.9 cells per section) compared with ovaries after 5 days of culture (549 ± 41.0 cells per section) (Figure 2C and D). Moreover, *Foxl2* mRNA and protein levels were found to be significantly lower in the Lenti-*Sp1*-sh group than in the control group after 5 days of culture (Figure 2E and F). These results indicate that SP1 determines the development of FOXL2+ pregranulosa cells to regulate PF formation.

SP1 governs the recruitment and maintenance of FOXL2+ pregranulosa cells

Recent studies have reported that FOXL2+ cells are derived from LGR5+ cells in the ovarian surface epithelium during PF formation (Ng et al., 2014; Rastetter et al., 2014; Feng et al., 2016); therefore, the number of FOXL2+ cells can be affected by

the proliferation of LGR5+ cells, the maintenance of FOXL2+ cells, or both processes. Based on the finding that SP1 is expressed in all somatic cells, the functional roles of SP1 in LGR5+ cells and FOXL2+ cells were studied. To determine whether SP1 governs the proliferation of LGR5+ cells, an *Lgr5* knockin (*Lgr5-KI*) reporter mouse model was used. *Lgr5-KI* mice were generated by integrating an enhanced green fluorescent protein (EGFP)-IRES-*Cre*ER[T2] cassette at the first ATG codon of the *Lgr5* gene (Barker et al., 2007). Therefore, LGR5 expression could be traced by staining with an anti-EGFP antibody, and proliferating cells were labeled with BrdU. The results showed that there were significantly fewer dividing LGR5+ cells in *Sp1* knockdown ovaries (12.0 ± 0.7 cells per section) than in control ovaries (40.0 ± 1.5 cells per section) after 5 days of culture (Figure 3A, arrows, and B). Thus, SP1 governs the recruitment of FOXL2+ pregranulosa cells by promoting the proliferation of LGR5+ cells.

In addition, to investigate the role of FOXL2+ cell-derived SP1 in regulating the maintenance of pregranulosa cells, we designed a vector encoding *Sp1* shRNA driven by the *Foxl2* promoter (F-*Sp1*-sh) that could specifically knock down *Sp1* expression in FOXL2+ cells. We injected F-*Sp1*-sh lentivirus or a scrambled control (F-SCR-sh) into fetal ovaries at 15.5 dpc and cultured them for 5 days or 8 days before examination. Immunostaining and western blot analysis revealed that SP1 protein levels were decreased specifically in somatic cells rather than in oocytes (Figure 3C and D). Furthermore, after 5 days of culture, the number of FOXL2+ cells in F-*Sp1*-sh-treated ovaries (638.0 ± 57.1 cells per section) was similar to that in control ovaries (729.5 ± 59.5 cells per section) (Figure 3C and E). However, in ovaries with specific *Sp1* knockdown, there were significantly fewer FOXL2+ cells in ovarian pregranulosa cells after culture for up to 8 days (437.0 ± 80.1 cells per section) than after culture for 5 days (638.0 ± 57.1 cells per section) (Figure 3C and E), although the number of FOXL2+ cells increased normally in control ovaries (1655.0 ± 55.2 cells per section). These results demonstrate that SP1 governs the maintenance of FOXL2+ cells during primordial folliculogenesis.

Pregranulosa cell-specific knockdown of Sp1 suppresses nest breakdown and oocyte apoptosis

Given that specific knockdown of *Sp1* in FOXL2+ cells dramatically decreased the number of pregranulosa cells, similar to *Sp1* knockdown in the entire ovary, we wondered whether FOXL2+ cell-specific knockdown of *Sp1* would also disrupt PF formation. Histological analysis (Figure 4A) and follicle number counting (Figure 4B and C) revealed a dramatic suppression of germline nest breakdown and PF formation in *Sp1* pregranulosa cell-specific knockdown ovaries compared with control ovaries after 5 or 8 days of culture (Figure 4B and C), demonstrating that SP1 expressed by pregranulosa cells plays a regulatory role in the formation of PFs. Furthermore, in *Sp1* pregranulosa cell-specific knockdown ovaries, relatively fewer loss of oocytes were observed after 8 days of culture (11328.0 ± 691.5 oocytes per ovary) than after 5 days of culture (15872.0 ± 680.5 oocytes
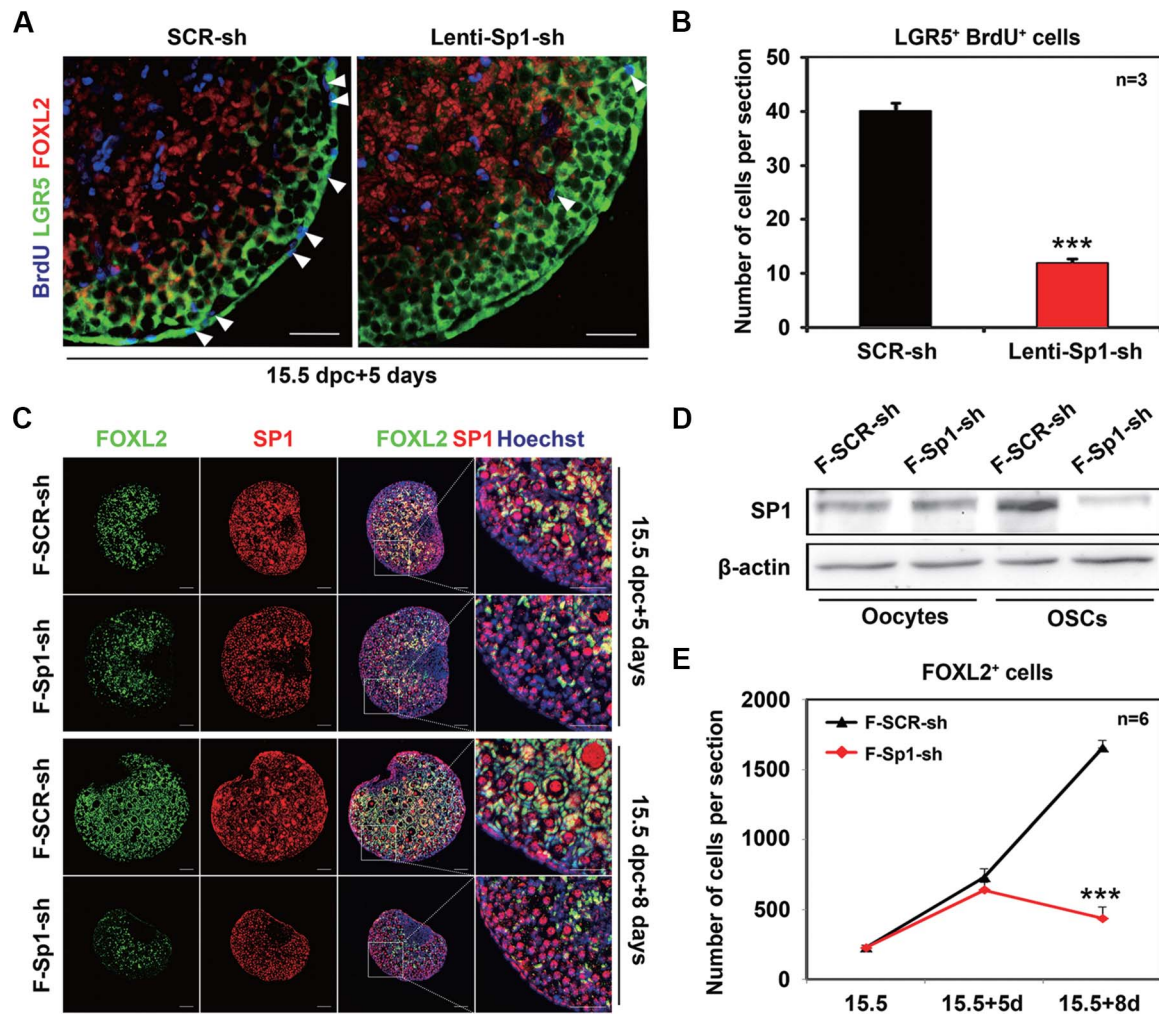
Figure 3 SP1 governs the recruitment and maintenance of FOXL2$^+$ cells. (**A** and **B**) Immunostaining (**A**) and quantitative analysis (**B**) show that *Sp1* knockdown significantly decreased the number of LGR5$^+$BrdU$^+$ cells and inhibited the proliferation of LGR5$^+$ progenitor cells. EGFP (representing LGR5): green; BrdU: blue; FOXL2: red. Arrowheads: LGR5$^+$BrdU$^+$ cells; scale bar, 40 μm. (**C**) Immunostaining of SP1 (red) and FOXL2 (green) in F-*Sp1*-sh-treated ovaries after 5 and 8 days of culture. Nuclei: Hoechst (blue). Scale bar, 40 μm. (**D**) SP1 protein levels were clearly lower in F-*Sp1*-sh OSCs than in controls after 5 days of culture. (**E**) Quantification of FOXL2$^+$ cells in F-SCR-sh and F-*Sp1*-sh-treated ovaries. The data are presented as mean ± SEM. ***$P < 0.001$ (*t*-test).

per ovary) (Figure 4B and C), unlike the large loss of oocytes in control ovaries after 8 days of culture (7532.0 ± 258.1 oocytes per ovary) compared with 5 days of culture (13952.0 ± 247.9 oocytes per ovary).

Given all the results obtained for *Sp1* knockdown in entire ovaries, we suspected that the germline nest breakdown arrest caused by the absence of pregranulosa cells suppressed the selective apoptosis of oocytes. To confirm this, a terminal-deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay was performed on *Sp1* pregranulosa cell-specific knockdown ovaries after 5 days of culture. The results showed that the number of apoptotic oocytes (Figure 4D, arrows) was significantly lower in *Sp1* pregranulosa cell-specific knockdown ovaries (9.3 ± 2.0 cells per section) than in control ovaries (27.3 ± 1.9 cells per section) (Figure 4E),

suggesting that the retention of oocytes in *Sp1* pregranulosa cell-specific knockdown ovaries was caused by reduced oocyte apoptotic cell death during the formation of PFs. Collectively, these results indicate that somatic-cell-derived SP1 governs PF formation as well as oocyte apoptosis by regulating the development of pregranulosa cells.

SP1 controls primordial folliculogenesis mainly through NOTCH2 signaling

Based on a report that NOTCH2 is required in somatic cells for the recruitment of pregranulosa cells as well as for the formation of PFs (Xu and Gridley, 2013; Vanorny et al., 2014), we investigated the relationship between SP1 and NOTCH2 signaling in the progression of PF formation. Immunofluorescence and western blot analysis showed that NOTCH2 was expressed in
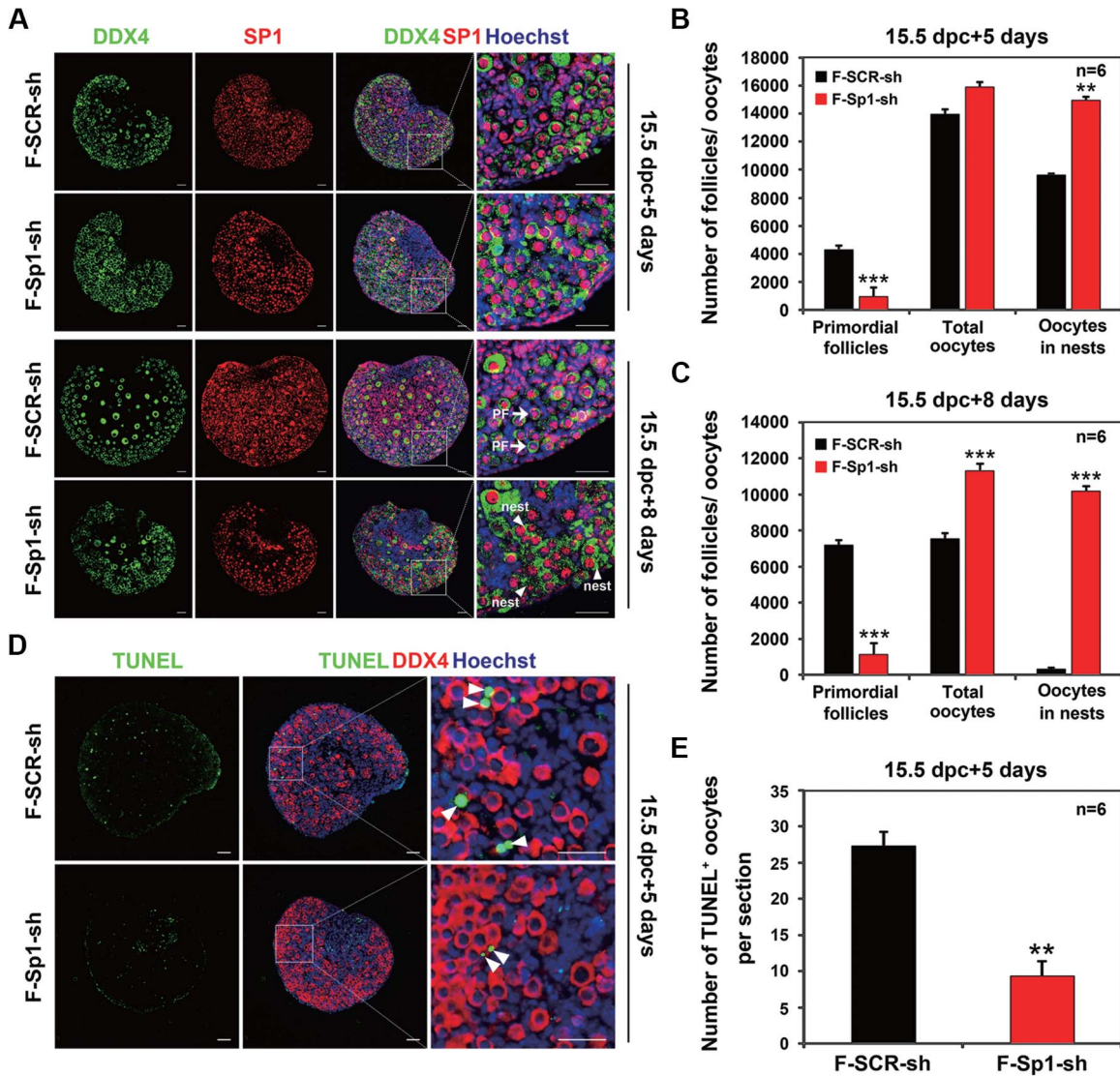
Figure 4 Pregranulosa cell-specific knockdown of *Sp1* suppresses nest breakdown and oocyte apoptosis. (**A**) The cell phenotypes and lentivirus infection efficiency in the ovaries were examined by SP1 (red) immunostaining after 5 and 8 days of culture. Oocytes were stained for DDX4 (green), and nuclei were dyed with Hoechst (blue). PF: arrows; nest: arrowheads; scale bar, 40 μm. (**B** and **C**) Quantification of the PFs, total oocytes, and oocytes in nests per ovary after 5 (**B**) and 8 days (**C**) of culture. (**D**) Representative micrographs showing TUNEL staining (green) in control and *Sp1* pregranulosa cell-specific knockdown ovaries. Oocytes in red (DDX4-positive); nuclear in blue (Hoechst). Arrowheads: TUNEL$^+$ oocytes; scale bar, 40 μm. (**E**) Quantification of TUNEL$^+$ oocytes. The data are presented as me3 ± SEM. **$P < 0.01$ and ***$P < 0.001$ (*t*-test).

somatic cells, and the protein levels of NOTCH2 increased during primordial folliculogenesis, consistent with the findings for SP1 in our experimental system (Supplementary Figure S5A and B). We further compared the influences of three treatments on PF formation, namely, knockdown of *Sp1* alone, inhibition of NOTCH2 alone by DAPT (a NOTCH signaling inhibitor), and inhibition of NOTCH2 plus knockdown of *Sp1*. The results showed that 8 days of DAPT treatment disrupted nest breakdown and PF formation (Supplementary Figure S5C and D), which was consistent with a previous report (Trombly et al., 2009). Additionally, ovaries with *Sp1* knockdown clearly had more total oocytes and oocytes

in nests than ovaries with NOTCH2 inhibition (Supplementary Figure S5C and D). In contrast, in *Sp1* knockdown plus NOTCH2 inhibition ovaries, the numbers of total oocytes and oocytes in nests were similar to those of ovaries with *Sp1* knockdown alone rather than ovaries with NOTCH2 inhibition alone. These results suggested that there was more severe nest breakdown failure in ovaries with *Sp1* knockdown than in ovaries with NOTCH2 inhibition. Coincidentally, although DAPT had no obvious effect on the expression levels of *Sp1* (Supplementary Figure S6A), knockdown of *Sp1* significantly reduced the expression of NOTCH2 and its target gene *Hey2* compared with the control treatment

(Figure 5A–C). Moreover, the levels of NOTCH2 were markedly increased in SP1-overexpressing ovaries compared with control ovaries (Figure 5D and E). These results indicate that NOTCH2 was the major downstream molecule of SP1, which was further supported by the dramatic decreases in both *Notch2* and *Hey2* expression levels in SP1 inhibitor-treated ovaries (Supplementary Figure S6B and C).

To confirm whether SP1 promoted primordial folliculogenesis in a NOTCH2-dependent manner, we overexpressed the active domain of NOTCH2 protein (NOTCH2 intracellular domain,

NICD2) in *Sp1* knockdown ovaries. First, western blot analysis showed that the NICD2 domain was successfully expressed in fetal ovaries, and accordingly, the mRNA levels of *Hey2* were markedly increased in NICD2-overexpressing ovaries, indicating that NOTCH2 was effectively overexpressed (Figure 5F and G). Further studies revealed that the protein levels of NOTCH2 were significantly restored in *Sp1* knockdown ovaries overexpressing NICD2 compared with control ovaries and *Sp1* knockdown ovaries without overexpression of NICD2 (Figure 5H). Consequently, overexpression of NICD2 in *Sp1* knockdown ovaries
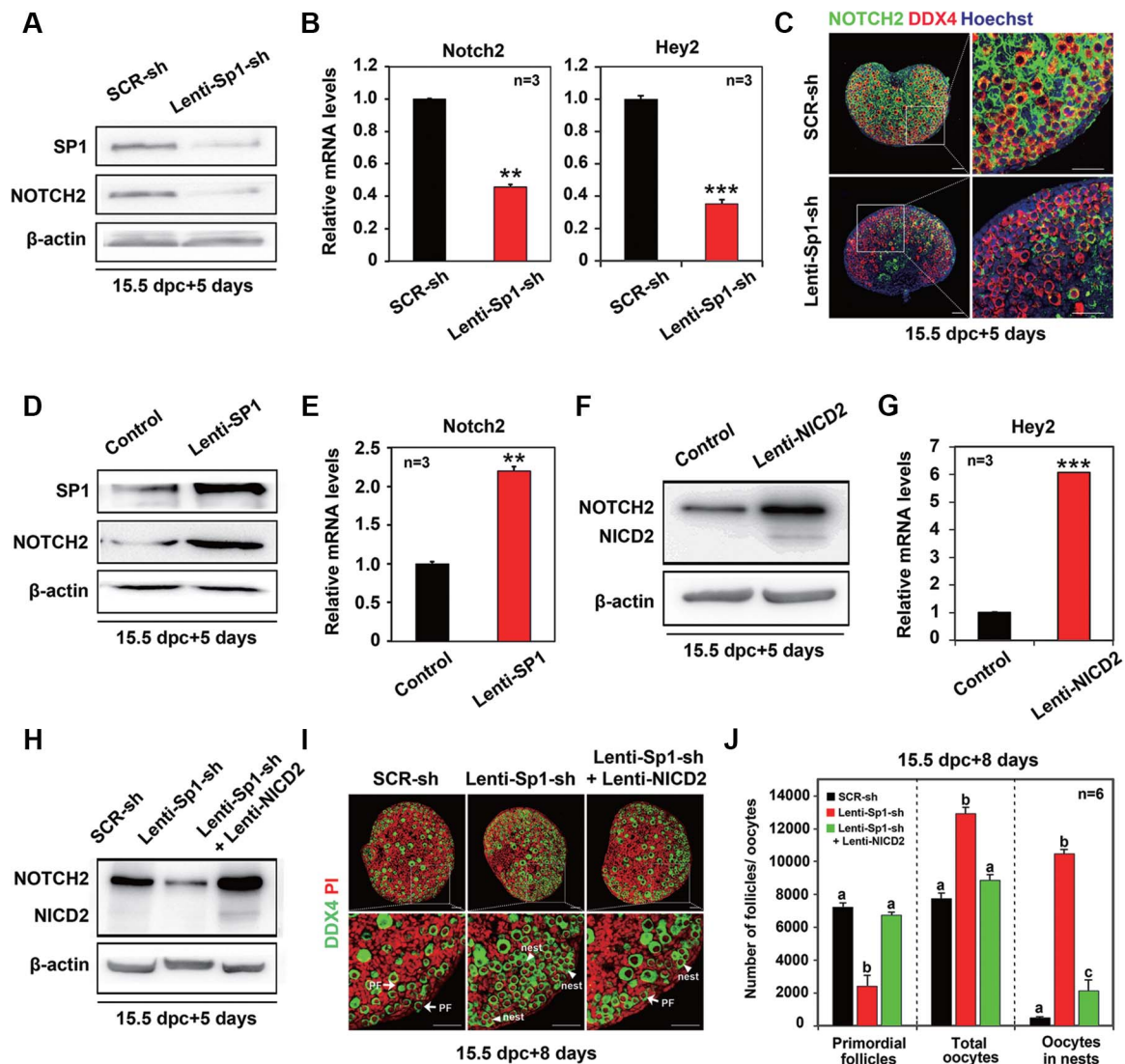

Figure 5 SP1 controls primordial folliculogenesis mainly through NOTCH2 signaling. (**A**) The NOTCH2 protein levels were assessed via western blot analysis. (**B**) The *Notch2* and *Hey2* mRNA levels were analyzed by quantitative real-time PCR. (**C**) Ovaries were immunolabeled for NOTCH2 (green) and DDX4 (red) and stained with Hoechst (blue). Scale bar, 40 μm. (**D and E**) The levels of SP1 and NOTCH2 were assayed by western blot analysis (**D**) and quantitative real-time PCR (**E**). (**F**) Western blot analysis of NICD2 domain expression. (**G**) The mRNA levels of *Hey2* in NICD2-overexpressing ovaries. (**H**) NICD2-overexpressing *Sp1* knockdown ovaries restored the protein levels of NOTCH2. (**I and J**) Immunostaining for DDX4 (green; **I**) and follicle number counting (**J**) after overexpression of NICD2 in *Sp1* knockdown ovaries. Nuclei were dyed with propidium iodide (red). Scale bar, 40 μm. The data are presented as mean ± SEM. **$P < 0.01$ and ***$P < 0.001$ (t-test); different letters (a–c) indicate significant differences between groups (ANOVA and Holm–Šidák test).

largely rescued the abnormalities in PF formation induced by *Sp1* knockdown, although a small portion of oocytes remained in nests (Figure 5I and J). These results demonstrate that NOTCH2-mediated primordial folliculogenesis is the major downstream event of SP1 signaling. However, other possible SP1 target genes involved in primordial folliculogenesis remain to be identified.

SP1 binds directly to the promoter of the Notch2 gene

To investigate the interactions and mechanisms underlying the SP1-mediated regulation of NOTCH2 expression, two assays were performed. First, a chromatin immunoprecipitation (ChIP) assay revealed that SP1 was able to bind to the promoter of the

Notch2 gene (in the region from −340 to −156) in both 16.5 dpc and 1 dpp ovaries; in addition, the binding ability in the 1 dpp group was much stronger than that in the 16.5 dpc group (Figure 6A). Second, a luciferase assay showed that cotransfection with SP1 (pCMV-SP1) and the *Notch2* promoter (*Notch2*-p) significantly enhanced luciferase activity compared with cotransfection with empty vector (pCMV) and *Notch2*-p (Figure 6B). Furthermore, the finding that the SP1 inhibitor treatment reversed the enhancement of luciferase activity only in the pCMV-SP1 and *Notch2*-p cotransfection group supported the idea that SP1 is recruited to the murine *Notch2* gene promoter region to activate *Notch2* expression (Figure 6B).
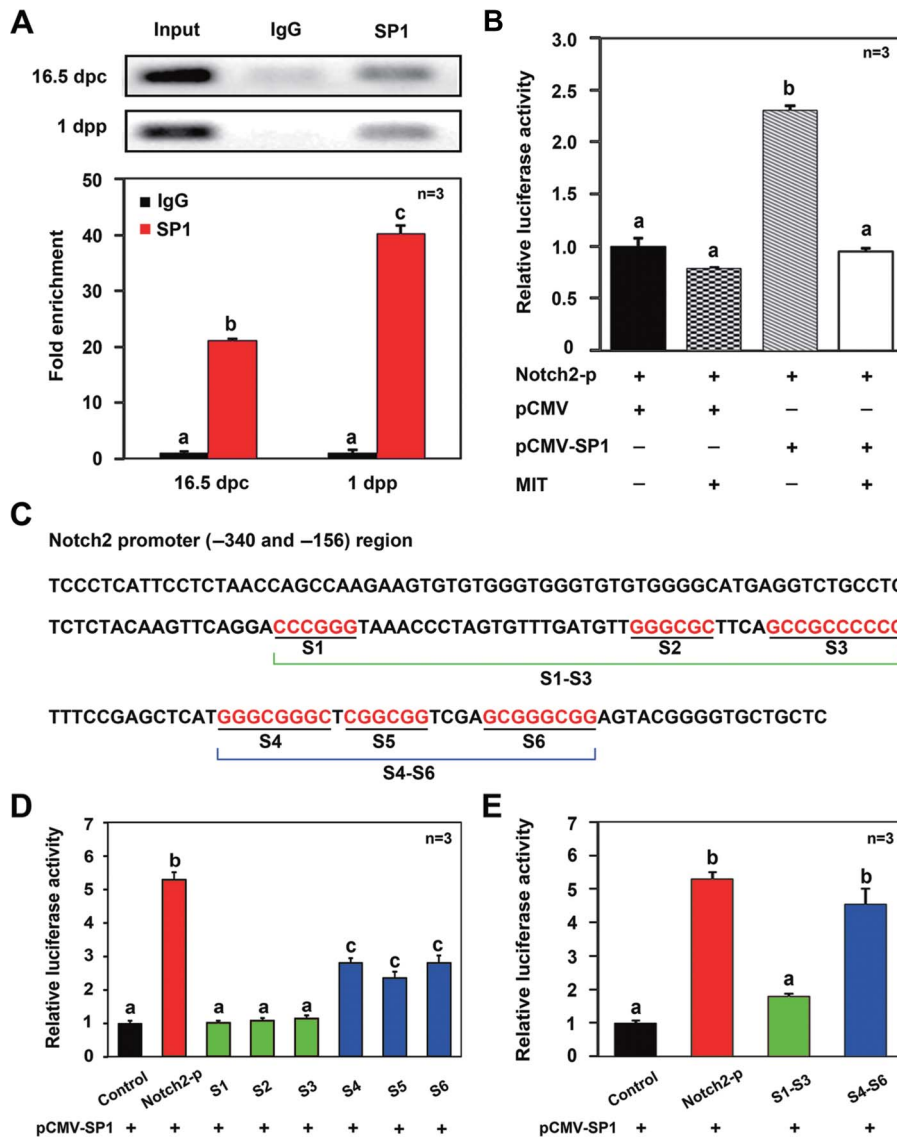

Figure 6 SP1 binds directly to the promoter of the *Notch2* gene. (**A**) A ChIP assay reveals that SP1 bound directly to the *Notch2* promoter. (**B**) Luciferase assay results showing that SP1 activated *Notch2* expression. *Notch2*-p, promoter of *Notch2* gene (the region from −340 to −156); PCMV-SP1, SP1-overexpressing vector; PCMV, empty vector. (**C**) The potential binding sites of SP1 on the *Notch2* promoter. (**D**) The ability of SP1 to bind to six GC-rich sequences on *Notch2*-p. (**E**) S4−S6, rather than S1−S3, comprised the SP1 binding site. The data are presented as mean ± SEM. Different letters (a−c) indicate significant differences between groups (ANOVA and Holm−Šidák test).

Given that most SP1-related diseases result from SP1 binding site mutations, it is necessary to identify the specific SP1 binding site on the *Notch2* gene promoter. Thus, the ability of SP1 to bind to six individual GC-rich sequences (named S1, S2, S3, S4, S5, and S6) in the *Notch2* promoter was tested (Figure 6C). The results showed that luciferase activity was ∼ 2.8-fold higher in the S4, S5, and S6 groups than in the control group. However, we did not observe increases in luciferase activity in the S1, S2, and S3 groups (Figure 6D). Nevertheless, the luciferase activity in the S4, S5, and S6 groups did not reach the same level as that observed in the *Notch2*-p group (Figure 6D), suggesting that each region is an indispensable portion of the SP1 binding site.

To investigate whether S4, S5, and S6 together constitute the entire SP1 binding site, the ability of SP1 to bind to the sequence from S4 to S6 (S4–S6) and the sequence from S1 to S3 (S1–S3) was examined (Figure 6C). As expected, the increase in luciferase activity in the S4–S6 group (but not in the S1–S3 group) was sufficient to attain the activity level in the group with the full-length SP1 binding region (*Notch2*-p), indicating that the S4–S6 sequence is the functional binding site for SP1 (Figure 6E). Taken together, SP1 directly promotes NOTCH2 expression by binding to the −199 to −173 region in the *Notch2* gene promoter.

FOXL2$^+$ cell deficiency leads to oocyte loss at prepuberty in Sp1 knockdown ovaries

Granulosa cell–oocyte interactions are well known to be critical for follicle survival and development (Fu et al., 2018). Although many more oocytes were reserved in nests in *Sp1* knockdown ovaries due to the absence of pregranulosa cells, we wondered whether these oocytes in nests were able to develop normally. Therefore, the developmental dynamics of lentivirus-infected ovaries were examined after 12 days of *in vitro* culture (equivalent to 7 dpp *in vivo*). Histological analysis showed that PFs (Figure 7A, arrows) and primary follicles (Figure 7A, arrowheads) were present normally in control ovaries; in contrast, in *Sp1* knockdown ovaries, a large portion of the oocytes and follicles had disappeared (Figure 7A). More importantly, some degenerating oocytes (Figure 7A, red frames) were observed in nests in *Sp1* knockdown ovaries, suggesting that the oocytes that remained in nests were unable to survive following ovarian development. Consistent with the essential role of SP1 in pregranulosa cell development during PF formation, FOXL2$^+$ cells were distributed in almost all somatic cells throughout the control ovaries after 12 days of *in vitro* culture; however, there were clearly fewer FOXL2$^+$ cells in *Sp1* knockdown ovaries (255.0 ± 52.2 cells per section) than in controls (2177.0 ± 108.3 cells per section) (Figure 7B and C). Only a few FOXL2$^+$ cells were present, which were mainly distributed around the remaining follicles; no follicles remained in the regions in which FOXL2$^+$ cells had disappeared (Figure 7B).

To demonstrate the developmental fate of *Sp1* knockdown ovaries under more physiological conditions, infected ovaries cultured for 5 days *in vitro* were transplanted under the renal capsules of 6-week-old female wild-type mice and maintained for an additional 2 weeks before examination. Histological analysis revealed that in control ovaries, most follicles had developed into secondary follicles (Figure 7D and E). In sharp contrast, in *Sp1* knockdown ovaries, all the oocytes in nests had disappeared, and very few oocytes were visible, similar to the phenotype of POI (Figure 7D and E). To further clarify whether the large amount of oocyte loss at prepuberty was due to the absence of granulosa cells in *Sp1* knockdown ovaries, the development of granulosa cells was examined via immunostaining for FOXL2 and NOTCH2. As expected, synchronous loss of FOXL2 and NOTCH2 expression was observed in the area where *Sp1* was silenced (Figure 7F). Moreover, large amounts of follicles disappeared in *Sp1* pregranulosa cell-specific knockdown ovaries, consistent with the phenotype of *Sp1* knockdown ovaries (Figure 7G). These results confirmed that SP1 mainly functioned in somatic cells to promote follicle formation and development.

Discussion

In this study, SP1-dependent pregranulosa cell development was shown to be pivotal for PF formation and survival in mice. SP1 controls the recruitment and maintenance of FOXL2$^+$ pregranulosa cells mainly in a NOTCH2-dependent manner. Furthermore, blocking *Sp1* specifically in pregranulosa cells suppresses oocyte apoptosis and results in the retention of large numbers of oocytes in nests perinatally. Due to the absence of functional granulosa cells supporting oocyte development in *Sp1*-silenced ovaries, all the oocytes in nests underwent cell death around puberty, resulting in a phenotype similar to POI. A possible working model of SP1 function is depicted in Figure 8. The findings indicate that SP1 is an indispensable TF that contributes to fertility.

Proper development of FOXL2$^+$ cells is essential for establishment of the PF pool (Maatouk et al., 2012; Mork et al., 2012; Pepling, 2012). Additionally, LGR5$^+$ cells in the ovarian surface epithelium have been demonstrated to be the origins of pregranulosa cells (Mork et al., 2012; Ng et al., 2014; Rastetter et al., 2014). Here, we investigated the functional role of SP1 in LGR5$^+$ and FOXL2$^+$ cells. On the one hand, using an *Lgr5-KI* reporter mouse model, we showed that SP1 governed the recruitment of pregranulosa cells by regulating the proliferation of LGR5$^+$ cells. On the other hand, knockdown of *Sp1* expression in FOXL2$^+$ cells significantly decreased the numbers of FOXL2$^+$ cells, demonstrating that SP1 also governs the maintenance of these cells. NOTCH signaling is known to be important for cell fate-determining processes, such as cell proliferation and differentiation, and especially for stem cell development in mammals (Chiba, 2006; Kovall et al., 2017). In addition, NOTCH signaling is involved in FOXL2$^+$ pregranulosa cell development and PF formation (Trombly et al., 2009; Feng et al., 2016). In particular, *Notch2* deletion in granulosa cells results in the formation of multioocyte follicles (MOFs) due to the persistence of oocyte nests (Xu and Gridley, 2013). However, the upstream signal that regulates Notch signaling in the perinatal ovary and its mech-
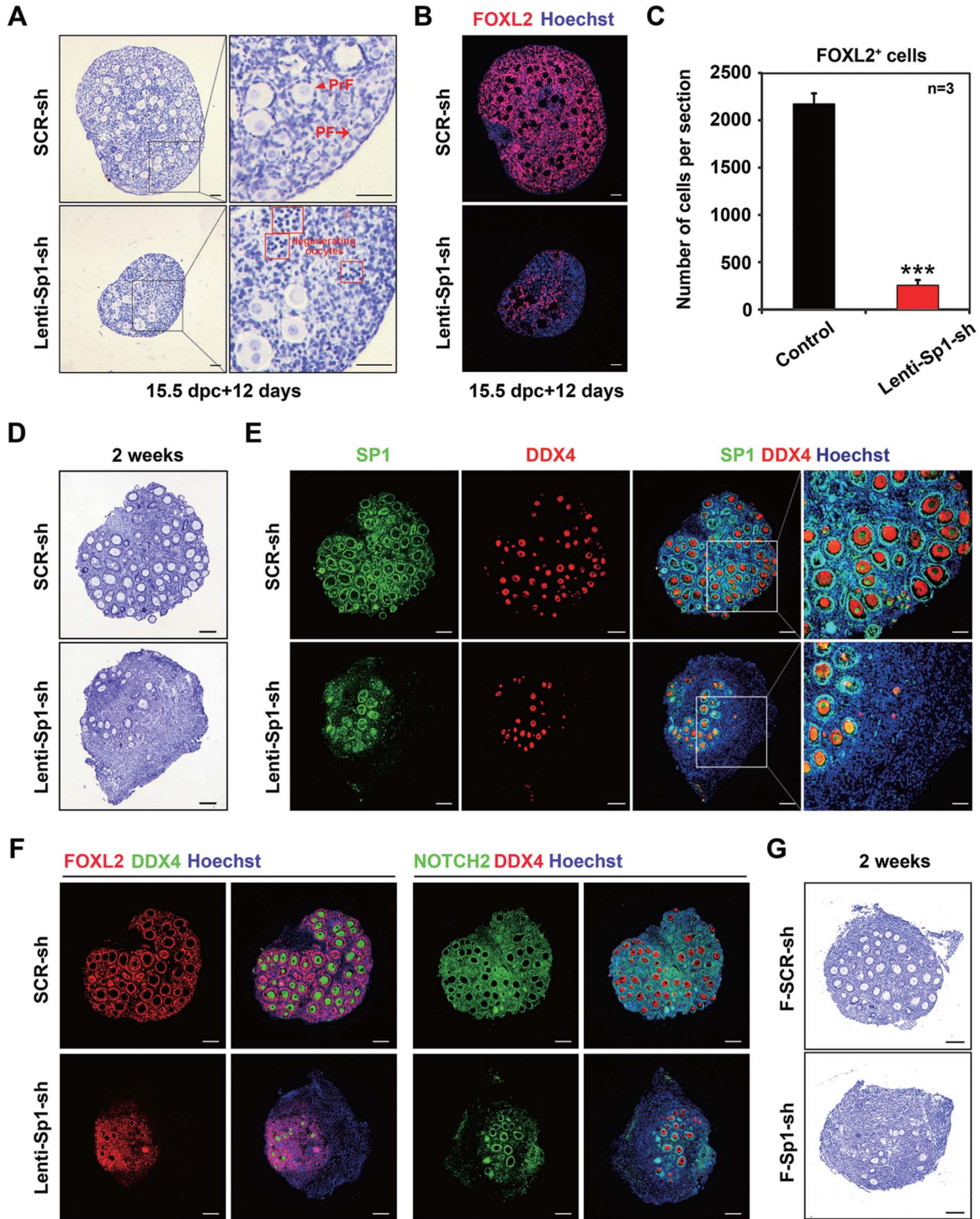
Figure 7 FOXL2$^+$ cell deficiency leads to oocyte loss at prepuberty in *Sp1* knockdown ovaries. (**A**) A large loss of oocytes and follicles in *Sp1* knockdown ovaries after 12 days of culture. PF: arrows; PrF (primary follicle): arrowheads; degenerating oocytes: red frames; scale bar, 40 μm. (**B** and **C**) FOXL2 immunofluorescence (red; **B**) and FOXL2$^+$ cell counting (**C**). Nuclei were dyed with Hoechst (blue). The data are presented as mean ± SEM. ***$P < 0.001$ (*t*-test). (**D** and **E**) Hematoxylin staining (**D**) and SP1 immunofluorescence (green; **E**) show that *Sp1* knockdown resulted in a severe loss of oocytes within nests in prepubescent ovaries. Oocytes in red (DDX4); nuclear DNA in blue (Hoechst). Scale bar, 140 μm. (**F**) Silencing of *Sp1* caused functional granulosa cell deficiency at prepuberty. FOXL2 (red) and DDX4 (green) (left); NOTCH2 (green) and DDX4 (red) (right). Nuclei in blue (Hoechst). Scale bar, 140 μm. (**G**) *Foxl2*-driven *Sp1* knockdown resulted in largely loss of oocytes in prepubescent ovaries. Scale bar, 140 μm.

anism of action are poorly understood. In this paper, we have provided evidence demonstrating that SP1 directs primordial folliculogenesis mainly through NOTCH2 signaling by directly binding to the promoter of *Notch2*. Other SP1 target genes involved in primordial folliculogenesis remain to be identified.

Generally, approximately two-thirds of oocytes undergo apoptosis at the time of PF formation *in vivo* (Baker, 1963; Hirshfield, 1991), although the underlying mechanism remains unclear. In this study, a TUNEL assay showed that apoptosis of oocytes in *Sp1* pregranulosa cell-specific knockdown ovaries was clearly suppressed, resulting in a 1.5-fold increase in total oocytes in the specific knockdown ovaries compared with control ovaries. These data indicate that SP1-mediated pregranulosa cell development controls the selective apoptosis of oocytes during PF formation. In agreement with this observation, membrane breakdown within nests and an apoptosis-like process are crosslinked with organelle transfer into oocytes (Lei and Spradling, 2016). In mice, well-developed oocytes with B-bodies are established within nests through organelle enrichment from

sister nest germ cells, the latter of which subsequently undergo apoptosis (Lei and Spradling, 2016; Wang et al., 2017a). However, inhibition of oocyte apoptosis within nests seems to have no effect on PF formation because when the *Bax* gene, an apoptosis-promoting factor, is deleted in newborn female mice, the number of PFs remains unchanged, although more oocytes are simultaneously available (Perez et al., 1999; Greenfeld et al., 2007). In contrast, studies by others and our group have indicated that nest breakdown and oocyte loss are interlinked perinatally (Edson et al., 2009; Lei and Spradling, 2016). In the *Sp1* conditional knockdown model, the persistence of germ cell nests and reduced oocyte apoptosis indicate the existence of a non-cell-autonomous effect on oocyte survival. Moreover, in prepubescent ovaries, a large loss of oocytes in nests occurred in both *Sp1* knockdown and *Sp1* pregranulosa cell-specific knockdown ovaries. This loss of oocytes could be a consequence of a shortage of functional granulosa cells to support follicle development. Additional studies are needed to elucidate the detailed mechanism, but these findings indicate that oocyte
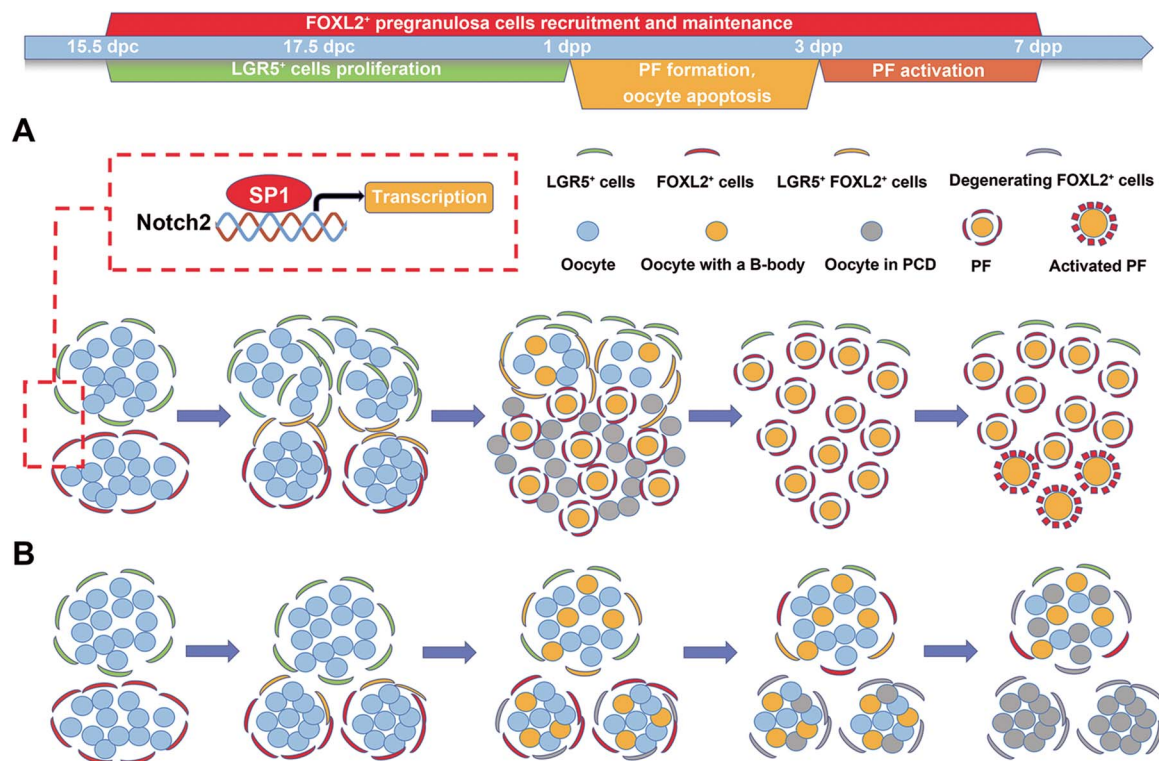

Figure 8 Schematic representation showing the proposed role of SP1 in PF formation. (**A**) Physiologically, the recruitment and maintenance of FOXL2+ pregranulosa cells are critical for PF formation. FOXL2+ cells are recruited from proliferating LGR5+ cells in the ovarian surface epithelium from 15.5 dpc to 1 dpp. Then, from 1 dpp to 3 dpp, the maintained FOXL2+ cells start to invade the nest germ cells and surround individual oocytes with B-bodies to form PF structures, while the rest of the oocytes undergo programmed cell death (PCD). From 3 dpp to 7 dpp, some PFs in the ovarian medulla are activated for further development. SP1 in LGR5+ cells and FOXL2+ cells plays an indispensable role in the recruitment and maintenance of pregranulosa cells by directly regulating *Notch2* transcription during PF formation. (**B**) In *Sp1*-silenced ovaries, the proliferation of LGR5+ cells and the maintenance of FOXL2+ cells are inhibited, which consequently decreases the number of FOXL2+ cells during PF formation and development. The deficiency in FOXL2+ cell development suppresses PF formation and causes large number of oocytes to remain in nests perinatally. However, due to the absence of a sufficient amount of functional granulosa cells to support oocyte development, all of the oocytes in nests undergo cell death following ovarian development (after PF formation at 3 dpp).

apoptosis could be a phenomenon initiated by pregranulosa cells surrounding individual well-developed oocytes to form PFs.

TFs, at least SP1 and FOXL2, are clearly actively involved in regulating ovarian development. However, these TFs seem to play different time-dependent roles in regulating folliculogenesis. In this study, knockdown of *Sp1* in fetal ovaries clearly decreased the recruitment and maintenance of pregranulosa cells, which caused a large number of nests to remain intact and caused few PFs to form. However, although FOXL2 was expressed very early in OSCs and is considered an important biomarker of the development of pregranulosa cells, loss of *Foxl2* has been found not to influence the formation of PFs (Schmidt et al., 2004). In contrast, following *Foxl2* deletion, pregranulosa cells do not change from flat to cubic shapes, indicating that these follicles cannot be activated to undergo further development (Schmidt et al., 2004). Therefore, SP1 expression may be a prerequisite for FOXL2 to function as a differentiation factor. Unfortunately, it is not clear whether direct regulation between SP1 and FOXL2 occurs under physiological conditions.

Generally, studies on genetically modified mice facilitate better understanding of targeted gene functions in biomedical research. To examine the role of SP1 in PF formation, *Sp1* conditional knockout mouse models can be used as alternative models, because systemic knockout of *Sp1* results in mouse death during the fetal stage (Marin et al., 1997). Because the process of primordial folliculogenesis occurs in such a narrow time window in perinatal mice, it is very hard to elaborately deplete *Sp1* specifically in OSCs during the appropriate time period due to the lack of satisfactory *Cre* mouse lines. On the one hand, depletion of *Sp1* too early may lead to oocyte death due to the critical role of SP1 in pregranulosa cell development, as demonstrated by the previous finding that disruption of the development of pregranulosa cells at 13.5 dpc leads to germ cell loss before birth (Padua et al., 2014). However, depletion of *Sp1* too late, for example, immediately after most PF formation has finished, is not suitable for investigation of its actions in our study. In fact, we produced two *Foxl2-Cre* lines of mice in our preliminary studies; unfortunately, neither of them exhibited satisfactory knockout efficiency during PF formation. Finding new lines to replace *Foxl2*-Cre mice remains difficult. Alternatively, *Sp1* knockdown mediated by siRNA or lentivirus can achieve depletion of *Sp1* during the critical period. In our studies, a lentivirus carrying *Sp1* shRNA was used for long-term knockdown of *Sp1* expression, especially in FOXL2+ cells, to mimic pregranulosa cell-specific depletion of *Sp1*. The results revealed that SP1 in somatic cells is crucial for pregranulosa cell development and PF formation.

In conclusion, we have supplied evidence that SP1 is involved in the recruitment and maintenance of pregranulosa cells during folliculogenesis. SP1 determines not only the dynamics of oocytes enclosed by pregranulosa cells but also the establishment and survival of follicles. These findings provide new insight into the *in vivo* mechanism of folliculogenesis and provide a foundation for the future establishment of *in vitro* follicle assembly using stem cell-derived germ cells.

Materials and methods

Animals

All wild-type mice were purchased from the Laboratory Animal Center of the Institute of Genetics and Developmental Biology (Beijing, China). The *Lgr5*-EGFP-IRES-*Cre*ER^T2 (*Lgr5-Kl*) reporter mice have been described in detail previously (Barker et al., 2007). Females (6–8 weeks old) were mated with males at a ratio of 1:1 overnight and checked for a vaginal plug the next morning. The time of discovery of a vaginal plug was designated 0.5 dpc. The day the pups were born was considered 0.5 dpp. All mice were maintained under a 16 h/8 h light/dark cycle at 26°C with free access to chow and water. The experiments were carried out in accordance with the principles and guidelines for the use of laboratory animals and approved by the Institutional Animal Care and Use Committee of China Agricultural University.

Ovary isolation and culture

Ovaries were separated from mouse ovarian capsules in prechilled PBS (10 mM, pH 7.4). The isolated ovaries were cultured in 6-well culture dishes (NEST Biotechnology) with 1.1 ml of basic DMEM/F12 (Gibco, Life Technologies) at 37°C in a 5% CO_2/95% air atmosphere with saturated humidity. Half of the medium was replaced every two days until the ovaries grew to the required stage. To determine the role of SP1 in primordial folliculogenesis, ovaries were treated with lentivirus containing shRNA (at 15.5 dpc) or with the SP1 inhibitor MIT (100 nM; M6891; Sigma-Aldrich) (at 16.5 dpc). For the BrdU incorporation assay, ovaries were treated with BrdU (B5002; Sigma-Aldrich) for 1 h before being collected for analysis.

Histological section analysis and follicle counting

Ovaries were fixed overnight at 4°C in 4% paraformaldehyde, embedded in paraffin, and serially sliced into 5-μm-thick sections. The sections were stained with hematoxylin, and the oocytes and follicles in every fifth section were counted. Follicles containing an oocyte surrounded by a single layer of granulosa cells with flattened nuclei were defined as PFs, and structures containing clusters of oocytes were defined as germ cell nests. To estimate the total number of oocytes and follicles in each ovary, the sum was multiplied by five, as described previously (Flaws et al., 2001).

Statistical analysis

All experiments were repeated at least three times, and the values are presented as the means ± SEM. The 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. Differences were considered statistically significant at $P < 0.05$.

More detailed methods are described in Supplementary material.

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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