Robo1/2 regulate follicle atresia through manipulating granulosa cell apoptosis in mice

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Secreted Slit proteins and their Roundabout (Robo) receptors act as a repulsive cue to prevent axons from migrating to inappropriate locations during the development of the nervous system. Slit/Robo has also been implicated in reproductive system development, but the molecular mechanism of the Slit/Robo pathway in the reproductive system remains poorly understood. Using a transgenic mouse model, we investigated the function of the Slit/Robo pathway on ovarian follicle development and atresia. We first demonstrated that more offspring were born to mice with a partial knockout of the Robo1/2 genes. We next showed that Robo1 and Robo2 are strongly expressed in ovarian granulosacells. Apoptosis in granulosa cells was reduced when Robo1/2 were partially knocked out, and this observation was further verified by in vitro Robo1/2 knockout experiments in mouse and human granulosa cells. We also found that ovarian angiogenesis wasenhanced by a partial lack of Robo1/2 genes. In summary, our data suggest that the Slit/Robo pathway can impact follicle development and atresia by influencinggranulosa cell apoptosis.

The ovaries are a pair of ductless female reproductive organs in which female germ cells are generated. The ovarian follicle is the fundamental functional unit of the ovary and is composed of oocytes, granulosacells, and thecal cells. Each primordial follicle has the potential to undergo folliculogenesis and to develop into a primary follicle, a secondary follicle, and finally intoa mature follicle that can ovulate, or degenerate in a manner similar to most other follicles that are not selected for maturation. At birth, the mammalian ovary contains numerous of primordial follicles, and these begin to undergo folliculogenesis during puberty and will eventually be ovulated or will degrade in a process known as atresia. The decision as to whether a follicle will develop or ovulate or undergo atresia is regulated by a variety of factors.

Slit was initially identified in Drosophila as a secreted protein that modulates the growth of glia cells at the midline during the development of the central nervous system. The receptor of Slit, Roundabout (Robo), is a transmembrane protein that is predominantly expressed on the axon growth cones in the central nervous system. Slit/Robo signaling is fundamental in the repulsion of axons away from the midline in both invertebrates and vertebrates and thus plays a key role in axon guidance at the midline of the central nervous system. Slit/Robo signaling is not restricted to the development of the nervous system, and it has also been shown to function in the development of the lung, kidney, and mammary gland. In addition to its physiological functions during embryonic development, Slit/Robo signaling has also been implicated in a variety of pathological conditions such as cancer and inflammation. The mammalian Slit family consists of Slit1, Slit2, and Slit3. All three proteins are expressed in the ventral neural tube during neurulation, but the expression patterns are not exactly the same. Slit1 is primarily expressed in the nervous system, but Slit2 and Slit3 are present in tissues other than the nervous system. The Robo receptor family consists of Robo1, Robo2, Robo3, and Robo4.

Dickenson et al. reported that Slit/Robo signaling also plays an important role in the reproductive system. The authors used RT-PCR to show that Slit2, Slit3, Robo1, Robo2, and Robo4 were expressed in sheep ovaries during gestation, and they demonstrated that the expression levels of Robo2 and Robo4 were elevated during the early stages of follicle formation and remained high throughout follicle maturation. More interestingly, Robo1 was found to be expressed in the pre-granulosacells, whereas Robo2, Robo4, and Slit2 were expressed in growing oocytes of the developing primordial follicle. Steroid hormones modulate Slit/Robo, which subsequently regulates reproductive
functions in the ovary and endometrium. However, the precise role of Slit/Robo in the regulation of physiological and pathophysiological ovarian functions remains poorly understood.

In this study, we aimed to assess the role of Slit/Robo signaling in ovarian follicle development and atresia using Robo1/2 transgenic mice. Based on the observed effect that Robo1/2+/− knockout affected fertility in mice, we carefully analyzed follicular development and atresia in Robo1/2-deficient mice and found a correlation with granulosal cell apoptosis and ovarian angiogenesis. Our experimental data suggest that Slit/Robo signaling might be involved in the regulation of folliculogenesis by regulating apoptosis in granulosa cells.

Methods

Transgenic mice. Robo1+/− and Robo2+/− double knockout transgenic mice(Robo1/2−/−, Strain Name: STOCK Robo1tm1Mtm Robo2tm1Mtm(=Mmrr) were purchased from the MMRRRC (Mutant Mouse Regional ResourceCenters, catalog, 030747-MU, https://www.mmrrc.org, San Diego, USA). The mice were genotyped by PCR. TheRobo1 primers wereF1: CGC GAC GAA GAT ATA TGT GC; F2: GAA GGA GGA CTG GTG GTT AGG; and F3: CCTCGGAACACTCTC ATTTCC. TheRobo2 primers wereF4: AAG TGC AAC GTC TCT GAA GTC CC; F5: GGC GCC GAA ATT CTT AAT TAA GCC GC; and F6: TTC TTT AGA AGG CAC AAC AAT CTC AGA G. For details regarding the genotyping protocols, please refer to the websites https://www.mmrrc.org/catalog/sds.php?mmrrc_id = 30747. Male wild-type mice were mated with Robo1/2−/− female mice. Robo1+/− and Robo2−/− mice are embryonic lethal. The mice were housed at 25 ± 1°C, 50% ± 10% humidity, and a 12 h:12 h light:dark cycle in a specific pathogen-free animal facility at the Animal Center, Guangdong Pharmaceutical University, Guangzhou, China. All methods were carried out in accordance with the approved guidelines and all animal experimental protocols were approved by the animal experimental ethics committee of Guangdong Pharmaceutical University.

Histology. Transgenic mouse ovaries were randomly collected from wild-type and Robo1/2−/− groups. Following immersion in 4% paraformaldehyde at 4°C for 12 h, the ovaries were embedded in paraffin wax. For hematoxylin and eosiin staining (H&E) and immunohistochemistry (IHC), the serial sections (5 μm) from each ovary were aligned in order on glass microscope slides. The follicles of each ovary from the different groups were then categorized and counted. The follicle clusters of each ovary were counted using an interactive platform with IPP6.0 software. The images of IHC and IF were analyzed using the CW4000 FISH Olympus software. For image analysis and scoring, a FluoroPlus Plus (IP60.0) professional imaging software was used. The positive-staining areas in the images were measured within an integrated optical density (IOD), and these values were used to analyze and compare all protein expression-positive cell count and TUNEL-positive cells.

In vitro fertilization experiment. The mice were injected with 10 U PMSG. After 48 h, 10 U hCG was administered. Following superovulation, eggs were collected from the ampulla of the uterine tube and co-cultured with wild-type mouse sperm. After 6−8 hours, the fertilized eggs were identified and evaluated. All sperm were obtained from the same male mouse.

Flow cytometry. Ovarian tissue from Robo1/2−/− knockout mice and wild-type mice were minced into 1 mm pieces to obtain granulosa cells and then 0.25% trypsin and 0.05% EDTA were added to complete the proteins and release the granulosa cells. The cells were collected at 800 rpm for 5 min. To investigate if Robo1 and Robo2 affect granulosa cell apoptosis, the Flow cytometry was performed according to the Annexin V/PI apoptosis assay kit protocol (Catalog: AP101-30, Multiscience Company, USA).

Statistical Analysis. The results are presented as the mean value (mean ± standard error). One-way ANOVA analyses and graphing were performed using the GraphPad Prism 5 software (GraphPad Software, CA, USA). The images of IHC and IF were analyzed with IPP6.0 software. Pearson’s Chi-square test was used to compare the Fertilization. The real-time PCR data were analyzed using Student’s t-test. A p-value less than 0.05 was considered significant.
Results

Robo1/2\(^{+/−}\) knockout increased fertility in mice. Dickinson et al. reported that Slit/Robo signaling was active during fetal ovary development and suggested that it might function in autocrine or paracrine interactions.\(^{13–15}\) Because of the embryonic lethality when Robo1/2 are knocked out \(^{19–20}\), Knocked out Robo1 and Robo2 both located on chromosome 16 as shown in Fig. 1A. Interestingly, we found that the number of offspring in the Robo1/2\(^{+/−}\) knockout mice was greater than the wild-type mice (Fig. 1B). To exclude any differences at different age, we analyzed the number of offspring at week 8 and week 16 and observed the same increase in offspring in the Robo1/2\(^{+/−}\) knockout mice at both time points (Fig. 1D). In addition, the weights of both female and male mice at 10 weeks of age were enhanced in Robo1/2\(^{+/−}\) knockout mice compared with the wild-type controls (Fig. 1D), but the mechanism behind this weight gain is unknown.

To further confirm this observation, Cy, a chemotherapeutic and immunosuppressive agent for the treatment of some neoplastic and autoimmune diseases (details in Materials and Methods), was administered to the wild-type and Robo1/2\(^{+/−}\) knockout mice at 4 weeks of age because Cy has been reported to disturb follicle growth and to result in premature menopause and sterility at high doses.\(^{16}\) The data shown in Supplementary figure 1A indicate that Robo1/2 knockout mice were able to rescue the Cy-induced reduction in offspring number. In the subsequent TUNEL assay with mice treated with Cy for 4 weeks, we found that the Robo1/2\(^{+/−}\) knockout could prevent the apoptosis that is induced by Cy treatment (Supplementary fig. 1C). We also calculated the number of various stages of follicles following apoptosis that is induced by Cy treatment (Supplementary fig. 1C).

Robo1/2\(^{+/−}\) knockout had a small impact on ovarian hormone secretion and gamete viability. To explore the potential mechanism behind the enhancement of fertility that was induced by Robo1/2\(^{+/−}\) knockout, we first assessed gameteviability by measuring the rates of ovum maturation and fertilization and the rates of zygote cleavage and degradation (Supplementary fig. 2). All determinations were performed in 4-week-old (Supplementary fig. 2A–B) and 10-week-old (Supplementary fig. 2E–F) mice, and we found no significant differences between the wild-type and Robo1/2\(^{+/−}\) knockout mice at either age.

Female fertility relies on the regulation of both pituitary and ovarian sex hormones. We measured the levels of prolactin, progesterone, estradiol, and testosterone in the blood of 10-week-old wild-type and Robo1/2\(^{+/−}\) knockout mice (Fig. 2). Unfortunately, we failed to successfully detect FSH and luteinizing hormone (LH), and this was most likely because of the difficulty in collecting sufficient amounts of mouse blood or the insensitivity for both FSH and LH. The results did indicate, however, that Robo1/2\(^{+/−}\) knockout led to an increase in progesterone secretion (Fig. 2A) but to no significant changes in the secretion of pituitary prolactin or ovarian estradiol or testosteron (Fig. 2B–D). So the findings suggest that the changes in fertility induced by Robo1/2\(^{+/−}\) knockout are probably not only the result of interference with hormone secretion.

Robo1/2\(^{+/−}\) knockout accelerated ovarian follicle maturation. One possible way to enhance fertility is to alter ovarian follicle development. Therefore, we compared the ovarian follicle development in the wild-type and Robo1/2\(^{+/−}\) knockout mice (Fig. 3). First, we found that the ovary weight dramatically increased in the Robo1/2\(^{+/−}\) knockout mice compared with the wide-type mice (data not shown). The general phenotype of the Robo1/2 knockout transgenic mice.

Figure 1 | The general phenotype of the Robo1/2 knockout transgenic mice. (A): The chart shows the Robo1 and Robo2 mutation sites in chromosome 16. (B): The photographs show the typical number of offspring in male WT crossbreed with Robo1/2\(^{+/−}\) knockout mice (female). (C): The chart shows the comparison of offspring number between WT (male) × WT (female) mice with WT (male) × Robo1/2\(^{+/−}\) knockout mice (female) (WT 8-week-old n = 10, Robo1/2 8-week-old n = 13; WT 16-week-old n = 19, Robo1/2 16-week-old n = 27). (D): The chart shows the weight comparison between the 10-week-old wild-type and Robo1/2\(^{+/−}\) knockout mice (WT female n = 7, Robo1/2 female n = 12, WT male n = 15, Robo1/2 male n = 19). ***p < 0.05 and **p < 0.001 indicate significant differences between the wild-type and Robo1/2\(^{+/−}\) knockout groups. Abbreviations: Chro, chromosome; WT, wild-type; Robo1/2\(^{+/−}\), double Robo1/2\(^{+/−}\) knockout.
Figure 2 | The hormone determination in wild-type and Robo1/2⁺⁻⁻ knockout mice. The progesterone, testosterone, prolactin, and estradiol levels in wild-type (WT, n = 8) and Robo1/2⁺⁻⁻ knockout (ROBO1/2⁺⁻⁻ knockout mice, n = 9) mouse blood were determined. (A): The blood progesterone levels in wild-type and Robo1/2⁺⁻⁻ knockout mice. (B): The blood testosterone levels in wild-type and Robo1/2⁺⁻⁻ knockout mice. (C): The blood prolactin levels in wild-type and Robo1/2⁺⁻⁻ knockout mice. (D): The blood estradiol levels in wild-type and Robo1/2⁺⁻⁻ knockout mice. *p < 0.05 indicates a significant difference between the wild-type and Robo1/2⁺⁻⁻ knockout groups.

Robo1 and Robo2 are robustly expressed in ovarian granulosa cells in wild-type mice. In the Robo1/2⁺⁻⁻ knockout mouse, we demonstrated that the lack of Robo1/2 increased the number of corpora lutea, and this suggests that ovulation was promoted by the down-regulation of Robo1/2 expression. In wild-type mice, the IHC analysis of Robo1 and Robo2 showed that both receptors are expressed in the ovary (Fig. 4A–B), especially in ovarian granulosa cells (Fig. 4A–A2, B1–B2). Figures 4C–E clearly show that Robo1 was expressed in the granulosa cells of the primary follicle (Fig. 4C), the granulosa cells in the secondary follicle (Fig. 4D), and the granulosa cells in the mature follicle (Fig. 4E). Figures 4F–H clearly show that Robo2 was also expressed in the granulosa cells of the primary follicle (Fig. 4F), the granulosa cells of the secondary follicle (Fig. 4G), and the granulosa cells of the mature follicle (Fig. 4H). To quantify the expression levels of Robo1 and Robo2 in the different stages of follicles, the IOD was calculated from the regions indicated by red dotted squares in Figures 4C–H. The results indicated that Robo1 is down-regulated in the different stages of follicles, and Robo2 is up-regulated in the different stages of follicles. These findings suggest that there is endogenous Robo1 and Robo2 expression in granulosa cells and that these receptors play a crucial role in the maintenance of follicle maturation and atresia.

Robo1/2⁺⁻⁻ knockout reduced apoptosis in ovarian granulosa cells. We measured changes in ovarian follicle development following the partial knockdown of Robo1/2. Apoptosis in the granulosa cells was closely associated with the dominant follicle selection and follicular atresia, thus we measured cell apoptosis in the Robo1/2⁺⁻⁻ knockout ovaries using a TUNEL assay (Fig. 5). Apoptosis in the granulosa cells of the Robo1/2⁺⁻⁻ knockout mice was dramatically reduced compared with the wild-type mice (Fig. 5A–B). The reduction in apoptosis induced by the partial knockdown of Robo1/2 was observed in follicles at various stages of development, including primary (Fig. 5A1–B1), secondary (Fig. 5A2–B2),
The total follicle number was also counted. Robo1/2 n induced by hyperstimulation are not apparently changed in the 4-week-old (B).

Figure 3 | The Robo1/2 knockout ovary promoted follicle maturation. (A): The two groups of ovaries from the 10-week-old wild-type and Robo1/2 knockout mice. (B): H&E staining of ovarian vertical sections from 4-week-old wild-type and Robo1/2 knockout mice. (C): H&E staining of ovarian vertical sections from 10-week-old wild-type and Robo1/2 knockout mice. (B’–C’): The diagrams show that the number of oocyte induced by hyperstimulation are not apparently changed in the 4-week-old (B’, WT n = 8, Robo1/2 n = 9) but increase in 10-week-old (C’, WT n = 6, Robo1/2 n = 3) Robo1/2 knockout mice, respectively. (D–E): Bar chart showing the changes in 4-week (D, WT n = 17, Robo1/2 n = 15) and 10-week (E, WT n = 8, Robo1/2 n = 10) ovarian follicle number in terms of follicle stage, including primary follicles, secondary follicles, and corpus lutea. The total follicle number was also counted. ***p < 0.001 indicates a significant difference between the wild-type and Robo1/2 knockout groups. Abbreviations: WT, wild-type; ROBO1/2/−/- mice, double Robo1/2 knockout. Scale bars = 200 μm in (A) and 500 μm in (B and C).

To further confirm these observations, we measured apoptosis in primary cultures of mouse and human granulosa cells (Fig. 6). We first performed the primary culture of the mouse granulosa cells that were isolated from the wild-type and Robo1/2 knockout ovaries, and we found some morphological differences between the wild-type and Robo1/2 knockout granulosa cells, the Robo1/2 knockout granulosa cells are more vigorously growth (Fig. 6A). And, the number of granulosa cells in the Robo1/2 knockout group was increased compared to the wild-type ovaries (Fig. 6B). Flow cytometry data showed that the reduced number of granulosa cells in the primary culture was a result of the decline in cell apoptosis as indicated by red squares in Fig. 6C and 6D. We next used primary cultures of human granulosa cells to confirm these observations (Fig. 6E, HE staining). The positive FSH receptor response in the IHC experiments confirmed these results because the FSH receptor is a marker for ovarian granulosa cells (Fig. 6F). Robo1 and Robo2 were down-regulated by siRNA and were not altered in the negative control using mock siRNA (Fig. 6G), and this down-regulation was associated with reduced cell apoptosis compared to wild-type (Fig. 6H–I). Thus, both in vivo and in vitro experiments indicated that cell apoptosis was reduced following the partial knockdown of Robo1/2 expression in ovarian granulosa cells.

Robo1/2 knockout increased ovarian angiogenesis. It has been well-established that apoptosis is involved in the biological process of follicular atresia in which the majority of follicles are eliminated while some follicles are elected as dominant follicles. This process is strictly regulated by FSH through the suppression of granulosa cell apoptosis. Therefore, a reasonable hypothesis is that the level of FSH in ovarian follicles should be related to angiogenesis in the ovaries, and well-known that Robo/Slit signaling are related to vascular. The CD34 antigen is present in immature hematopoietic precursor cells, so we examined its expression in ovarian tissue. We found that Robo1/2 knockout increased ovarian angiogenesis. The same localization images of CD34 expression in the primary, secondary, and mature follicles compared with wild-type (Fig. 7A–C). Pericytes are known to stabilize blood vessels and the α-SMA antigen is present in pericytes, we used α-SMA to further assess the effects of Robo1/2 knockout on angiogenesis. The same localization images of the ovaries showed that α-SMA-positive cells in the Robo1/2 knockout (Fig. 7D–F) significantly increased CD34 expression in the primary, secondary, and mature follicles compared with wild-type (Fig. 7A–C). The increase in angiogenesis occurred in the primary, secondary, and mature follicles (not all data shown). The immunofluorescent staining for CD31, which is normally expressed on endothelial cells, also indicated more small blood vessels in the Robo1/2 knockout ovary (Fig. 7G) compared with the wild-type ovary (Fig. 7I). The IHC data suggest that ovarian angiogenesis was promoted by the Robo1/2 knockout in the mouse ovary.
Discussion
Slit/Robo signaling exerts its effects during tissue morphogenesis. Thus, the disruption of certain Slit and/or Robo proteins is often associated with tumor formation in different tissues. Dickinson et al. demonstrated that Slit/Robo signaling could also perform crucial functions in the reproductive system. The expression of Slit/Robo in the pre-granulosa cells and the oocytes of the developing primordial follicle indicate that Slit/Robo signaling might function through both autocrine and paracrine interactions. To further explore the role of Slit/Robo in reproductive biology, we generated a partial knockout of the Robo1/2 genes in mice because full knockout was embryonically lethal. We found that the partial lack of Robo1/2 resulted in a greater number of offspring compared with the wild-type mice. Weyers et al. demonstrated that Slit/Robo signaling is pivotal for proper gonad formation, although gonad formation has been found to be regulated by multiple and independent pathways. Thus, we have examined in vitro gamete vitality by measuring the rates of ovum maturation and

Figure 4 | Robo1 and Robo2 are primarily expressed in ovarian granulosa cells. (A–B): The immunocytochemistry against Robo1 (A) and Robo2 (B) was performed on the vertical sections of the ovaries. (A1–A2): The high-magnification images of Robo1 expression as indicated by the dotted squares in (A). (B1–B2): The high-magnification images of Robo2 expression as indicated by the dotted squares in (B). (C–E): The high-magnification images of Robo1 expression in a primary follicle, a secondary follicle, and a mature follicle. (F–H): The high-magnification images of Robo2 expression in a primary follicle, a secondary follicle, and a mature follicle. (I): The bar chart showing the comparison of integral optical density (IOD) for Robo1 expression in primary (n = 10), secondary (n = 5), and mature (n = 5) follicles. (J): The bar chart showing the comparison of the IOD for Robo2 expression in primary (n = 8), secondary (n = 5), and mature (n = 5) follicles. *p < 0.05 and **p < 0.01 indicate significant differences between the WT and Robo1/2 knockout groups. Scale bars = 200 µm in (A–B) and 20 µm in (A1–A2), (B1–B2), and (C–H). Negative control group: mouse IgG (K, L, M).
are able to mature and eventually ovulate in each estrous cycle, i.e., granulosa cells are indispensable for the normal development of intimately correlated with follicle maturation and atresia because matured (Fig. 4C).

Robo2 genes were strongly expressed in the ovarian granulosa cells knocked out in mice, but in wild-type mice both the Robo1 and SCIENTIFIC REPORTS Skating influences fertility.

Figure 5 | Apoptosis in granulosa cells is reduced in Robo1/2+/− knockout mice. (A–B): The detection of apoptosis by the TUNEL assay was performed on the vertical sections of ovaries obtained from wild-type (A) and Robo1/2+/− knockout (B) mice. (A1–A3): The high-magnification images from a wild-type ovary (A) showing TUNEL staining in a primary follicle (A1), a secondary follicle (A2), and a mature follicle (A3). (B1–B3): The high-magnification images from a Robo1/2−/− knockout ovary (B) showing TUNEL staining in a primary follicle (B1), a secondary follicle (B2), and a mature follicle (B3). (C): The bar chart showing the percentage of TUNEL-positive apoptotic granulosa cells in primary (n = 3), secondary (n = 3), and mature (n = 3) follicles from the wild-type and Robo1/2−/− knockout mice. (D–E): Immunochemistry against caspase-3 was performed on vertical sections of the wild-type (D) and Robo1/2−/− knockout (E) ovaries. (D1–E1): The high-magnification images from the sites indicated by red squares in (D and E), respectively. **p < 0.01 and ***p < 0.001 indicate significant differences between the wild-type and Robo1/2−/− knockout groups. Abbreviations: WT, wild-type; ROBO1/2−/− mice, double Robo1/2−/− knockout. Scale bars = 500 μm in (A–B) and 20 μm in (A1–A3) and (B1–B3).

The results indicated that there were no significant alterations in gamete viability following the partial knockout of the Robo1/2 genes. Furthermore, Cy treatment normally leads to ovarian follicle exhaustion21, but the Robo1/2−/− knockout could prevent this (Supplementary Fig. 1A), and this suggests that normal Slit/Robo signaling might promote follicle atresia via effects on granulosa cell apoptosis (Supplementary Fig. 1C). This finding also verified the hypothesis that Slit/Robo signaling influences fertility.

In the subsequent analysis of follicle development, we determined that the increase in offspring number might be closely related to the development of activated follicles (Fig. 3). Thus, Robo1/2−/− knockout increased follicle maturity and decreased follicle atresia (Fig. 3E), and this indicated that more offspring were born to mice with a partial lack of Robo1/2 genes. In general, this observation is consistent with previous reports in which over-expression of Slit/Robo in follicular atresia that granulosa and lutein cells undergo apoptosis by transformation into the corpora lutea. It is during the process of follicular atresia that granulosa and lutein cells undergo apoptosis (Supplementary Fig. 1C). This finding also verified the hypothesis that Slit/Robo signaling influences fertility.

The TUNEL assay demonstrated that apoptosis in the granulosa cells from the Robo1/2−/− knockout follicles was substantially reduced compared with those from wild-type follicles (Fig. 5A–B), and apoptosis was reduced in all stages of the developing follicles (Fig. 5C). The reduction in apoptosis induced by Robo1/2−/− knockout was verified by IHC against caspase-3 (Fig. 5D–E), which is another marker for apoptosis. This is because granulosa cells, which comprise the layer of small cells that form the wall of the ovarian follicle, are fundamental in determining the follicle’s fate. At the same time, the influence of Robo on apoptosis is again the important factor that supports our hypothesis in this study. Therefore, we double-checked the effects of Robo1/2 knockout in vitro mouse and human granulosa cell cultures (Fig. 6). The in vitro experimental assay demonstrated that Robo1/2−/− knockout reduced the apoptosis-positive cell population (Fig. 6C), and this was also reflected in the greater number of cells in the Robo1/2−/− knockout mouse granulosa cell culture (Fig. 6B). In the human granulosa cell culture, there were fewer apoptotic cells when Robo1/2 was partially knocked out using Robo1/2-siRNA compared with the cells receiving mock siRNA. Thus the experimental results from the in vivo and in vitro Robo1/2−/− knockout were the same, and we conclude that partial lack of Robo1/2 leads to a reduction of granulosa cell apoptosis.

fertilization and the rates of zygote cleavage and degradation in the 4-week-old (Supplementary Fig. 2A–D) and 10-week-old (Supplementary Fig. 2E–H) wild-type and Robo1/2−/− knockout mice. The results further following the formation of an antrum. Only the selected follicles become ovulatory follicles, and ovulation is then followed by transformation into the corpora lutea. It is during the process of follicular atresia that granulosa and lutein cells undergo apoptosis.
Riaz et al. reported that the somatostatin receptor 2 sub-type regulates granulosacell apoptosis and proliferation through selective constitutive action that is independent of somatostatin25. Wnt signaling has also been demonstrated to negatively regulate follicular development via components of the Foxo3a signaling pathway26. The proliferation and aromatization capacity of rat granulosacells are stimulated by both FSH and TGF-beta27. FSH has been demonstrated to regulate granulosacell proliferation through its influence on micro-RNA expression. From previously published studies, we can reasonably speculate that the determination of follicle fate (to undergo either maturation or atresia) relies on the levels of FSH or other hormones. Thus, the levels of FSH or other hormones delivered...
by ovarian angiogenesis most likely play a crucial role in the determination of follicle fates. Thus, we can evaluate the hormone level, such as FSH, through the assessment of the local angiogenesis in ovaries if we cannot directly measure FSH levels in the follicles. In addition, the interaction between the secreted Slit ligand and Robo receptor has been implicated in the regulation of cell death and angiogenesis. It is not surprising, therefore, that ovarian angiogenesis was detected by IHC against CD34 and IF staining against SMA and CD31 (Fig. 7). The angiogenesis data demonstrated that Robo1/2 knockout could lead to an increase of angiogenesis around developing follicles (Fig. 7G, J). These findings imply that our hypothesis regarding the hormone level alterations induced by Robo1/2 knockout has an amorphological basis in angiogenesis. Further investigation will be required to explore the mechanism for how Robo1/2 knockout can affect angiogenesis in ovaries. And another question is whether or not higher level of progesterone in Robo1/2 knockout is associated with less follicle apoptosis.

In summary, we have used a transgenic mouse model to demonstrate the potential role of the Slit/Robo signaling pathway in the reproductive capacity in mice. The hypothesis is illustrated in Fig. 8. The Slit/Robo pathway might be involved in the regulation of ovarian follicle development and atresia by targeting the granulosa cells for apoptosis. Another potential pathway is most likely the effect on ovarian angiogenesis, which alters FSH and other hormone levels. These hormone levels, in turn, modulate granulosa cell proliferation and apoptosis. Therefore, upon induction of Robo1/2 knockout,
more mature follicles form and less follicular atresia occurs and this leads to a greater number of offspring being born to mice with a partial lack of Robo1/2. Further molecular biological experiments are needed to better understand the role of the Slit/Robo pathway in reproductive biology.

Figure 8 | A proposed model of the potential mechanisms of Slit/Robo signaling in the regulation of ovarian follicle atresia. (A): The Robo1/2 knockout would decrease the granulosa cell apoptosis and angiogenesis, which lead to follicles atresia. Figure B and C be used to show Robo1/2 knockout results in more number of mature follicles.

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Author contributions
X.S.Y. and L.J.W. wrote the manuscript. J.C.L., Y.X.Y., R.L.Z., L.L.Z., X.W.H., G.W. and D. H. performed the experiments and prepared the figures. J.Y.C. and X.D.H. collected data and managed the transgenic mice. All authors reviewed the final manuscript.

Additional information
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