WNT signaling in pre-granulosa cells is required for ovarian folliculogenesis and female fertility

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ABSTRACT
In mammalian ovaries, immature oocytes are reserved in primordial follicles until their activation for potential ovulation. Precise control of primordial follicle activation (PFA) is essential for reproduction, but how this is achieved is unclear. Here, we show that canonical wingless-type MMTV integration site family (WNT) signaling is pivotal for pre-granulosa cell (pre-GC) activation during PFA. We identified several WNT ligands expressed in pre-GCs that act in an autocrine manner. Inhibition of WNT secretion from pre-GCs/GCs by conditional knockout (cKO) of the wntless (Wls) gene led to female infertility. In Wls cKO mice, GC layer thickness was greatly reduced in growing follicles, which resulted in impaired oocyte growth with both an abnormal, sustained nuclear localization of forkhead box O3 (FOXO3) and reduced phosphorylation of ribosomal protein S6 (RPS6). Constitutive stabilization of β-catenin (CTNNB1) in pre-GCs/GCs induced morphological changes of pre-GCs from a squamous into a cuboidal form, though it did not influence oocyte activation. Our results reveal that canonical WNT signaling plays a permissive role in the transition of pre-GCs to GCs, which is an essential step to support oocyte growth.

KEY WORDS: Reproduction, WNT signaling, Folliculogenesis, Oogenesis, Granulosa cells, Oocytes, Mouse

INTRODUCTION
In female mammals, including humans, precise control of folliculogenesis is essential for fertility. Oocytes are protected and grow within follicles, which are the fundamental units of the ovary. Dormant oocytes are arrested at the diplotene stage of meiosis I, reserved in primordial follicles, and surrounded by pre-granulosa cells (pre-GCs) (Pepling, 2006; Pepling and Spradling, 2001). Only a small proportion of primordial follicles is activated concurrently, with activation resulting in follicular growth and the serial development of growing follicles, which resulted in impaired oocyte growth with both an abnormal, sustained nuclear localization of forkhead box O3 (FOXO3) and reduced phosphorylation of ribosomal protein S6 (RPS6). Constitutive stabilization of β-catenin (CTNNB1) in pre-GCs/GCs induced morphological changes of pre-GCs from a squamous into a cuboidal form, though it did not influence oocyte activation. Our results reveal that canonical WNT signaling plays a permissive role in the transition of pre-GCs to GCs, which is an essential step to support oocyte growth.

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Wiese et al., 2018). Whereas WNT4-mediated canonical WNT signaling has been shown to be important for sex determination during embryonic development (Parma et al., 2006; Vainio et al., 1999), the function of WNT signaling during postnatal folliculogenesis remains unclear. Both Wnt2 knockout and GC-specific Wnt4 knockout female mice were found to have slightly reduced fertility, with the mild nature of this defect in each case likely being due to functional redundancy among WNT ligands (Boyer et al., 2010; Monkley et al., 1996). More recently, oocyte-derived R-spondin 2 (RSPO2) was shown to contribute to the activation of WNT signaling in GCs (De Cian et al., 2020). RSPO2 is a WNT agonist that is secreted extracellularly and enhances canonical WNT signaling (Kazanskaya et al., 2004), and follicle growth was found to be impaired in ovaries with loss of RSPO2 function in transplant experiments (De Cian et al., 2020). Although WNT signaling is implicated together with other important factors such as GDF9 (growth differentiation factor 9) and BMP15 (bone morphogenetic protein 15) in PFA (Dong et al., 1996; Dube et al., 1998), its mechanism of action has been unknown. Here, we reveal an essential role of canonical WNT signaling in regulation of the transition of pre-GCs to mature GCs during PFA by focusing on postnatal folliculogenesis and taking advantage of mouse mutants that avoid the issue of the redundancy of WNT ligands.

RESULTS

Canonical WNT signaling in pre-GCs is essential for female fertility

Although WNT signaling has been implicated in adult folliculogenesis, the spatiotemporal patterns of WNT ligand expression in the mouse ovary have been insufficiently characterized (Harwood et al., 2008). Both the complexity and specificity of WNT signaling in mice are due in part to the expression of 19 WNT ligands. To identify the specific WNT ligands that are expressed during folliculogenesis, we performed in situ hybridization analysis with ovaries from 3-week-old wild-type (WT) mice for all 19 Wnt mRNAs (Fig. S1). Among the 19 WNT ligands, the mRNAs for Wnt4, Wnt6 and Wnt11 were detected in the GC lineage from the primordial follicle to primary follicle stages (Fig. 1A). The abundance of these WNT ligand mRNAs gradually declined in association with the transition to preantral follicles. Minimal expression of Wnt2, Wnt2b, Wnt9a, Wnt5b, Wnt11 and Wnt16 was observed in the oocytes of primordial follicles (Fig. 1A; Fig. S1). These findings suggest that pre-GC/GC-
derived WNT signals might contribute to the early stages of folliculogenesis.

To examine the effects of attenuated WNT signaling, we generated ovarian somatic cell-specific Wntless conditional knockout (Wls cKO) mice by crossing Sfi-Cre mice [which express Cre recombinase under the control of the steroiogenic factor 1 gene (Sfi; Nr5a1 promoter)] to mice harboring a ‘floxed’ (Wls\textsuperscript{floxed}) and a ubiquitous deletion (Wls\textsuperscript{del}) allele of Wls (Carpenter et al., 2010; Dhillon et al., 2006). Given that WLS is required for secretion of all WNT ligands, the resulting Sfi-Cre;Wls\textsuperscript{floxed} (Wls cKO) mice allow us to examine the effects of inhibiting WNT ligand secretion specifically from ovarian somatic cells, including GC and theca-lineage cells, from embryonic day (E) 11.5 (Dhillon et al., 2006; Piprek et al., 2019). Sfi-Cre;Al9 reporter mouse ovaries were used to compute Cre recombination efficiency in FOXL2-positive GC lineage cells, which was 98.30 ±0.72% at P0 and 97.37±1.08% at 2 weeks of age (mean±s.e.m.). We found no Cre activity in oocytes. In Wls cKO mice, no obvious morphological abnormalities were apparent during development through adulthood (Fig. 1B). To obtain Wls cKO mice, female Wls\textsuperscript{floxed} and male Sfi-Cre;Wls\textsuperscript{del} mice were mated. The male: female ratio of the resulting litters was 1.10 (n=500) overall and 1.14 (n=137) for the Wls cKO mice, with no significant difference using the chi-squared test. The birth rate of all Wls cKO mice was 27.4%, which is close to the theoretical rate of 25.0%. The ovaries of Wls cKO mice were similar to those of littermate control mice at postnatal day (P) 0, whereas they manifested atrophy at 2 weeks of age (Fig. 1B). To evaluate reproductive performance, we housed 8-week-old control or Wls cKO female mice (n=7 per genotype) with WT males for 24 weeks. Wls cKO females were completely infertile (Fig. 1C) even though they engaged in spontaneous mating behavior.

To identify WNT-active cells, we evaluated the ovaries of a WNT signal reporter mouse line, R26-WntVis. The green fluorescent protein (GFP) reporter activity of these mice reflects the activity of the canonical WNT signaling pathway (Takeimoto et al., 2016). GFP was specifically expressed in the GC lineage from the primitive to primary follicle stages (Fig. 1D), consistent with the expression pattern of Wnt mRNAs (Fig. 1A). FOXL2 was examined as a marker for pre-GCs/GCs and DDX4 as a marker for oocytes in this analysis. The WntVis signal was also sparsely detected in the interstitial cells, theca cells and ovarian epithelium, but not in blood vessels (Fig. S2A-E). It was undetectable in oocytes (Fig. 1D). The WntVis signals were most abundant and intense in pre-GCs of primordial follicles, and they became less abundant and less intense with follicle growth (Fig. 1D-F). In the control group, the median was 60.5% for WntVis-positive cells in the pre-GC population that were composed of primordial follicles (Fig. 1E). As primordial follicles contain several pre-GCs, this is expected that most primordial follicles are receiving Wnt signaling to some extent. Both the number of WntVis-positive pre-GCs/GCs and WntVis fluorescence intensity were significantly reduced in Wls cKO mice harboring the R26-WntVis allele compared with control mice (Fig. 1D-F). Together, these results thus suggested that autocrine WNT signaling activity in pre-GCs is required for female fertility.

**WNT signaling is required for the pre-GC to GC transition and subsequent development**

To investigate the cause of the ovarian defects of Wls cKO mice, we performed a more detailed morphological analysis (Fig. 2A). Immunostaining of DDX4 revealed that the number of oocytes per ovary did not differ significantly between Wls cKO and control mice at P0 (Fig. 2C). Periodic acid-Schiff staining with hematoxylin (PAS-H) revealed few atypical follicles, such as those containing multiple oocytes, in the ovaries of the mutant females at 2 weeks of age (Fig. 2B). Abnormal sexual differentiation was not apparent, as confirmed by sex genotyping (Fig. S3). These data suggested that germ cell survival during embryonic development and sex determination were not affected in Wls cKO mice. Whereas cuboidal GCs were apparent in growing follicles containing oocytes with a diameter of 20-40 μm in control mice, flattened and morphologically abnormal GCs were detected in Wls cKO mice (Fig. 2B). In contrast, no morphological abnormalities were detected in primordial follicles with an oocyte size of <20 μm (Fig. 2B). Quantitative analysis revealed that the GC layer was significantly thinner in growing follicles of Wls cKO mice, whereas it was similar in primordial follicles of both genotypes (Fig. 2D,E). GCs in Wls cKO mice were less likely to become multilayered, and even when they did form multiple layers, the layers were uneven (Figs 2B and 3A). To describe the morphology of GCs further, we categorized growing follicles by the appearance of GCs as squamous, cuboidal and columnar (Table S1). Within the growing follicles, primary follicles with squamous GCs were 26.2% in Wls cKO mice, notably higher than the 6.6% observed in controls. Secondary/preantral follicles with columnar GCs with distinct cell polarity were not observed in Wls cKO mice, but 48.4% were found in controls. Secondary/preantral follicles in Wls cKO mice mainly consisted of cuboidal type cells (27.9%). Overall, these results indicated that the PFA-associated transition of pre-GCs to GCs is suppressed in the absence of WNT signaling, yet GCs of some follicles can proceed to the cuboidal stage.

To assess whether WNT signaling might trigger PFA, we quantified the number of follicles per ovary and categorized them by follicle type as primordial (Pm), activated primordial (APF: oocyte diameter of >20 μm without cuboidal GCs), primary (Pr), or secondary-antral (Sec-Ant) at 2 weeks of age. The number of primordial follicles in Wls cKO mice was similar to that in control mice (Fig. 2F), suggesting that WNT ligands are not a triggering stimulus but rather a permissive signal for PFA; otherwise, the accumulation of primordial follicles in the mutant ovaries would have been expected. The observation that oocytes larger than 20 μm were present in the ovaries of Wls cKO mice (Fig. 2D,E) also suggested that these cells are capable of initiating a growth response to PFA. However, in contrast to control ovaries, the ovaries of 2-week-old Wls cKO mice lacked oocytes with a diameter of 45-60 μm. Oocytes with a diameter of >45 μm constituted 26.8±6.2% of all oocytes in control females but only 0.5±0.5% of those in Wls cKO females (P=0.0286, nonparametric Mann–Whitney matched-pairs test) (Fig. 2D). The retardation of oocyte growth in Wls cKO mice therefore appeared to occur between PFA and full maturity. The number of developing follicles was significantly lower in Wls cKO mice (Fig. 2F), with insufficient GC maturation likely giving rise to follicular atresia.

WNT signaling plays an important role in female sex determination during embryogenesis (Parma et al., 2006; Vainio et al., 1999). We therefore next examined the effects of postnatal deletion of Wls with the use of the Wt1\textsuperscript{CreERT2} knock-in allele (Zhou et al., 2008). Control and Wt1\textsuperscript{CreERT2};Wls\textsuperscript{floxed} (PN-Wls cKO) mice were injected with tamoxifen at P3, P5 and P7 to induce Wls\textsuperscript{del} deletion and were studied at 3 weeks of age (Fig. 2G). Wt1\textsuperscript{CreERT2};Ai9 mice showed 99.5±0.09% (mean±s.e.m.) efficiency of the Cre recombination within FOXL2-positive cells at 3 weeks of age after tamoxifen administration. The phenotype of PN-Wls cKO female mice appeared to be essentially identical to that of Wls cKO females. The PN-Wls cKO mice thus showed morphologically normal primordial follicles and attenuated transition of pre-GCs to GCs in growing follicles (Fig. 2H,I). Primary follicles with squamous GCs made up 35.6% of the follicles counted in PN-Wls cKO mice whereas only 2.4% were
found in controls at 3 weeks of age (Table S2). Hence, the defect found in the pre-GC to GC transition of Wls cKO and PN-Wls cKO mice is not the result of disrupted cell fate determination during embryogenesis, but rather a result from the lack of WNT signaling during folliculogenesis. The number of primordial follicles in PN-Wls cKO mice was also similar to that of control mice (Fig. 2J), providing further evidence that initiation of PFA can take place without WNT signaling. The ovaries of PN-Wls cKO mice to determine the effects of postnatal deletion of Wls on folliculogenesis in ovarian somatic cells. (H) PAS-H staining of ovarian sections from tamoxifen-treated 3-week-old PN-Wls cKO mice. (I) GC layer thickness categorized by oocyte diameter for follicles of 3-week-old PN-Wls cKO mice (n=239 follicles from six control mice; n=315 follicles from six PN-Wls cKO mice). Horizontal lines represent the median. **P<0.01, ***P<0.001 (unpaired multiple t-tests with the Holm-Sidak correction). (J) Quantification of follicle number per ovary for 3-week-old PN-Wls cKO mice as determined by immunohistochemical staining for DDX4. Data are mean+s.e.m. (n=7 mice per genotype). ***P<0.001 (unpaired multiple t-tests with the Holm-Sidak correction).

Functional impairment of the transition of pre-GCs to GCs gives rise to insufficient oocyte activation

To evaluate whether GCs in Wls cKO mice are functionally mature, we assessed the expression of anti-Müllerian hormone (AMH), a marker for GCs. Pre-GCs of primordial follicles initially do not express AMH. AMH becomes expressed once GCs grow and transition to a cuboidal/columnar morphology and then is released into the circulation (Visser et al., 2006). In Wls cKO mice, however, immunofluorescence staining revealed only a low level of AMH expression in GCs (Fig. 3A). PFA is hypothesized to be a locally regulated process, whereas the later stages of folliculogenesis are influenced markedly by GC-derived paracrine factors and gonadotropins (Sánchez and Smitz, 2012). We therefore next analyzed major GC-derived hormones (AMH, inhibin A and estradiol) and gonadotropins [follicle-stimulating hormone (FSH) and luteinizing hormone (LH)] in order to shed light on GC function and the endocrine system in Wls cKO mice. The concentrations of AMH and inhibin A in serum were significantly lower in Wls cKO mice than in controls at 8 weeks of age (Fig. 3B,C). These data indicated that GC function is markedly suppressed in Wls cKO mice. Estrogens are primarily produced by developing follicles to coordinate systemic reproductive functions (Hillier et al., 1994; Miller and Auchus, 2011), but in this study their urinary concentration did not differ between the two genotypes (Fig. 3D). Given that estrogen production to some extent has been reported in
mutant mice lacking sexual maturation, ovariectomized mice and human patients whose ovarian steroidogenesis is inhibited (De Tassigny et al., 2007; Miller and Auchus, 2011; Saito et al., 2009), Wls cKO mice may also possess a compensatory mechanism that allows for estrogen production. By contrast, the serum levels of FSH and LH were significantly higher in Wls cKO mice than in control mice (Fig. 3E,F), possibly reflecting a positive feedback response to the suppressed follicle development and lack of ovulation in the mutant females. Pituitary gland function may thus be normal in Wls cKO females, even though Sf1-Cre is expressed in endocrine glands (Dhillon et al., 2006). Importantly, low Amh and high FSH levels in serum are considered diagnostic criteria for human POI (Jankowska, 2017; Méduri et al., 2007).

GC proliferation is a key contributor to follicle growth, and the mTOR signaling pathway, which is implicated in PFA, appears to regulate GC proliferation (Yu et al., 2011). To assess the role of
other signaling pathways such as WNT signaling in GC proliferation, we examined Wls cKO mice by performing immunostaining for MKI67 (Ki67) and measuring the signal intensity for all FOXL2-positive pre-GCs/GCs within follicles. Although the percentage of MKI67-positive GCs increased with follicle growth in both control and Wls cKO mice, the increase was less pronounced in the mutant animals (Fig. 3G). Most pre-GCs of primordial follicles were negative for MKI67 in both control and Wls cKO mice (Fig. 3G). Transzonal projections (TZPs) are membranous extensions from GCs that pass through the zona pellucida to the oocyte cell membrane and are important for normal oocyte development (Albertini et al., 2001; Carabatos et al., 1998). Staining of filamentous actin with Phalloidin revealed the absence of obvious TZP structures in Wls cKO ovaries (Fig. 3H). These results thus indicated that the abrogated folliculogenesis of Wls cKO mice is attributable to impaired GC proliferation and the inability of GCs to support oocyte growth.

Although we found that oocyte growth is initiated in Wls cKO mice, it was unclear whether the oocytes undergo normal activation. To evaluate oocyte status, we analyzed the expression of FOXO3, a transcription factor that contributes to maintenance of oocyte dormancy, by quantifying the nuclear to cytoplasmic ratio of its immunofluorescence intensity (Castrillon et al., 2003). In control mice, whereas primordial follicles manifested a nuclear FOXO3 localization, FOXO3 was exported from the nucleus during PFA (Fig. 3J). However, in Wls cKO mice, both oocytes with a diameter of <20 µm and those with a diameter of 30-40 µm showed a higher FOXO3 intensity in the nucleus than in the cytoplasm (Fig. 3J). In both primary and secondary follicles, FOXO3 was localized in the nuclei of growing oocytes of Wls cKO mice (Fig. 3L). FOXO3 is known to be phosphorylated by the PI3K-AKT pathway (John et al., 2008), whereas phosphorylation of the ribosomal protein S6 (RPS6) is a key downstream event of the PI3K-AKT-mTOR pathway in PFA (Adhikari et al., 2009; Reddy et al., 2008). RPS6 contributes to oocyte growth by promoting protein translation and ribosomal biogenesis. Phosphorylated-RPS6 is not obvious in the oocytes of primordial follicles, but becomes evident once they are activated. In Wls cKO mice, phosphorylated-RPS6 in activated oocytes is markedly suppressed (Fig. 3K,L). This result suggested that the delay in oocyte growth in Wls cKO mice (Fig. 2D) is likely due to insufficient function of RPS6. Collectively, these data indicated that, even if oocytes increase in size, they do not undergo the normal activation process in Wls cKO mice. These results further suggested that activated GCs are necessary for proper oocyte growth; the functions of FOXO3 and RPS6 via the overlapping PI3K-AKT pathway are under the influence of GCs. We then examined the expression levels of GC-derived KITL and its receptor KIT, which CTNNB1 or the WNT agonist RSPO1 was forcibly expressed (Bourboun et al., 2005, 2006; De Cian et al., 2017).

A WNT activator rescues the phenotype of Wls cKO mouse ovaries in vitro
To verify the phenotype of Wls cKO mice, we determined the effects of a WNT inhibitor in ovarian culture. Ovaries isolated from WT mice at P4 were maintained on membrane cell culture inserts for 6 days by the gas-liquid interphase method, either in the presence of dimethyl sulfoxide (DMSO) as a vehicle control or the WNT inhibitor IWP2, which blocks porcupine O-acyltransferase (PORCN)-mediated palmitoylation and consequent secretion of WNT ligands (Chen et al., 2009) (Fig. 5A). The ovaries at the end of the culture period thus corresponded to ovaries at P10 in vivo. PAS-H staining revealed that IWP2 markedly suppressed GC layer development, whereas it had only a minimal effect on primordial follicles with an oocyte diameter of <20 µm (Fig. 5B,C). We then

The pre-GC layer is expanded by a dominant stable form of CTNNB1
To investigate whether WNT signaling is sufficient for the pre-GC transition to GCs, we generated Wt1CreERT2;Ctnnb1lox(ex3)By (CTNNB1-CA) mice, which express a stable form of CTNNB1 in the somatic lineage of ovaries (Fig. 4A). The stabilized CTNNB1 binds to T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors, which activate the expression of target genes for canonical WNT signaling (Harada et al., 1999). Ovaries of (tamoxifen-treated) CTNNB1-CA mice were similar in size to those of control mice at 3 weeks of age, but were more spherical in appearance and had a smoother surface compared with control ovaries (Fig. 4B). The observation that somatic cells were densely packed in the ovarian interstitium of CTNNB1-CA mice suggests that hyperproliferation of interstitial cells was responsible for these differences. Morphological abnormalities were not apparent in GCs of growing follicles in the mutant mice, whereas pre-GCs of primordial follicles were not squamous but cuboidal (Fig. 4B). An increase in pre-GC layer thickness was also detected in primordial follicles containing oocytes with a diameter of <20 µm in CTNNB1-CA mice (Fig. 4C), and MKI67 immunostaining revealed that the proliferation of pre-GCs in primordial follicles was enhanced (Fig. 4D). Follicles with oocytes of <20 µm and with four or fewer pre-GCs/GCs that showed no obvious columnar shape were classified as primordial follicles in the mutant ovaries. These results thus revealed that Wnt signaling promotes the transition of pre-GCs to GCs. Of note, CTNNB1-CA mice showed a normal subcellular localization pattern for FOXO3 in their oocytes (Fig. 4E). Given that we believe that WNT plays only a permissive role in PFA, it was not surprising that localization of FOXO3 in oocytes was not affected by hyperactivation of canonical WNT signaling in GCs. However, the expression levels of KITL and KIT were slightly reduced in CTNNB1-CA (Fig. S4C), in contrast to the Wls cKO phenotypes. As WNT and KIT signaling are fundamental pathways to regulate the pre-GC transition to GC and oocyte growth, respectively, it is possible that these two pathways can mutually adjust their activity in order to balance the PFA outcome, an idea that can be tested in future studies.

Quantification of follicle number revealed no depletion of primordial follicles or increase in the number of developing follicles in CTNNB1-CA mice (Fig. 4F), suggesting that CTNNB1 stabilization (activation of canonical WNT signaling) is insufficient for induction of PFA. Inhibition of follicle growth was apparent in the mutant mice, however, with the number of secondary/preantral follicles being significantly reduced (Fig. 4F), and GCs secondary/preantral follicles were less proliferative (Fig. 4D). Constitutive activation of WNT signaling likely influences GCs, interstitial cells and theca cells in such a manner that the survival and growth of growing follicles are impaired. These characteristics are consistent with the reduced proliferative capacity and canecular changes of GCs previously observed for mice in which CTNNB1 or the Wnt agonist RSPO1 was forcibly expressed (Boerboom et al., 2005, 2006; De Cian et al., 2017).
cultured ovaries from Wls cKO or control mice with the WNT activator CHIR99021 in an attempt to rescue the phenotype of the mutant ovaries (Fig. 5D). CHIR99021 activates the canonical WNT signaling pathway by inhibiting glycogen synthase kinase 3 (GSK3) and thereby stabilizing CTNNB1 (Bennett et al., 2002). CHIR99021 induced a significant thickening of the GC layer at all assessed follicular stages in both control and Wls cKO ovaries (Fig. 5E,F). Of note, the abnormal flattened morphology of GCs in Wls cKO ovaries was completely normalized by CHIR99021 treatment (Fig. 5E). These data indicated that the function of WNT signaling in folliculogenesis was evident in vitro, and that a WNT activator was able to promote follicle growth in a commonly adopted culture system.

The mTOR signaling pathway is implicated in PFA. Nutritional or other factors are thus thought to activate mTOR signaling in pre-GCs and thereby stimulate the production of KITL required for oocyte activation (Liu et al., 2014). Given that WNT signaling has been shown to activate mTOR complex 1 (mTORC1) as a result of inhibition of GSK3 (Inoki et al., 2006), we investigated the potential role of WNT signaling as an upstream regulator of mTOR signaling in GCs. The addition of an activator of mTOR signaling, 3BDO, to ovarian cultures induced a significant increase in GC layer thickness in follicles of Wls cKO and control mice (Fig. 5G-I). However, this rescue effect for Wls cKO ovaries was limited, even in growing follicles (Fig. 5H,I). These data suggested that WNT and mTOR signaling contribute to PFA in a coordinated manner, rather than through a simple hierarchical relationship.

**DISCUSSION**

In this study, we propose a postnatal function of canonical WNT signaling to permit the transition of pre-GCs to GCs in an autocrine manner, which is required for facilitating oocyte growth (Fig. 6). Without WNT signaling, the majority of the GC population manifested characteristics similar to pre-GCs, including a squamous shape, hypoproliferative state, limited production of AMH and lack of TZP formation. WNT-mediated pre-GC to GC transition appears to couple PFA with the nuclear-cytoplasmic shuttling of FOXO3 and phosphorylation of RPS6 in oocytes to exit from dormancy. As neither attenuation nor enhancement of WNT signaling contribute to PFA in a coordinated manner, rather than through a simple hierarchical relationship.
taking into consideration the fact that oocytes are able to complete maturation and attain their full size only with the support of GCs, the activation of which is dependent on WNT signaling.

Our results reveal that WNT signal activation occurs exclusively at the primordial follicle stage. WNT signaling in cuboidal/columnar GCs is likely detrimental to folliculogenesis, given that forced activation of canonical WNT signaling in pre-GCs/GCs reduced the number of developing follicles. Our data are consistent with the previous finding that activation of WNT signaling induced abnormal follicle growth with increased GC apoptosis in an in vitro culture of secondary follicles (Li et al., 2014). The activation of WNT signaling specifically at the primordial follicle stage is likely achieved as a result of the characteristic expression pattern of Wnt4/6/11, which is strongly expressed in the primordial to primary follicle stage, followed by a weaker expression in GCs of preantral follicles as they grow. Because WNT signaling activation occurs during a narrower time window than when the Wnt ligands are expressed, additional mechanisms may regulate the timing of WNT signaling activation. For example, production of functional RSPO2 by oocytes is important for the activation of canonical WNT signaling in GCs (De Cian et al., 2020). Given that Rspo2 mRNA was found to be abundant in oocytes of growing follicles, a mechanism likely exists to inhibit WNT signaling after the primary follicle stage. BMP15 has been identified as an inhibitor of WNT signaling during early embryogenesis in *Xenopus* (Di Pasquale and Brivanlou, 2009), and activated mouse oocytes begin to secrete BMP15 at the primary follicle stage (Dube et al., 1998), which makes BMP15 a candidate mediator of WNT signaling in growing follicles. WNT signaling in pre-GCs/GCs may thus be precisely controlled at several levels, including the spatiotemporal specificity of Wnt ligand expression and the production of RSPO2 and BMP15 by oocytes.

It was reported that WNT4/RSPO1 initiates ovarian differentiation by activating the canonical WNT signaling pathway at the embryonic stage (Chassot et al., 2014). *Wnt4* is expressed in the gonads of both sexes until E11.5; it is then repressed in the male gonads and becomes ovary-specific from E12.5 onward. Although partial gonadal sex reversal has been
reported in Wnt4 and Rspos null mice (Parma et al., 2006; Vainio et al., 1999), no such phenotype was observed in the Wls cKO mice used in this study. It is possible that the suppression of WNT secretion may have occurred later than gonadal sex determination, as there is likely a delay between the initial expression of Cre recombinase, the deletion of the target Wls gene and the suppression of WNT protein secretion. As SF1 expression in somatic cells of female gonads is known to be attenuated after E12.5 (Ikedo et al., 1994), Wls cKO under Sf1-Cre control may have progressed gradually until birth. It should be noted that the Cre expression starts from E11.5 in the Sf1-Cre line we used (Dhillon et al., 2006; Piprek et al., 2019), whereas another Sf1-Cre, which is often used in studies of sex differentiation, is reported to be expressed from E10.5 onward (Bingham et al., 2006). Recently Cheng et al. reported GC-specific conditional deletion of Wls by using Amhr2-Cre that initiates Cre expression from E13.5 (Cheng et al., 2020). They reported impaired luteinization of Amhr2-Cre; Wlslox/fox mice, although no abnormalities in sex determination were noticed, which is consistent with the present study. It is possible that Cre-mediated recombination of the Wls gene may not be highly efficient, which might explain the absence of sex reversal and the phenotypic difference between Amhr2-Cre; Wlslox/fox and Sf1-Cre; Wlslox/del (homozygous for fox) and Sf1-Cre; Wlslox/del (heterozygous for fox and null). We successfully showed the role of postnatal WNT signaling by generating PN-Wls cKO mice, but the fetal phenotype of Wls cKO ovaries needs to be scrutinized further. As Sf1-Cre is expressed in the theca cell lineage as well as pre-GC/GCs, the potential role of theca cell-derived WNTs on pre-GC development and PFA should be addressed in follow-up studies using theca cell-specific Cre mice.

Recent progress in the field of in vitro gametogenesis (IVG) has had a great impact on reproductive biology and medicine (Hikabe et al., 2016; Hamazaki et al., 2021). Fully developed oocytes can now be obtained from embryonic stem cells or induced pluripotent stem cells of mice by the application of IVG techniques. Although the protocol for stem cell-derived oocytes for fertility treatment in humans has not yet been fully established, vigorous research is underway (Yamashiro et al., 2018). Simultaneously, in vitro activation (IVA) has recently been described as an innovative method of fertility treatment for women with POI (Kawamura et al., 2013; Suzuki et al., 2015; Zhai et al., 2016). Wnt-related genes have not been identified as genes responsible for POI, but we have now shown that the hormonal environment of Wls cKO mice is similar to that of women with POI (De Vos et al., 2010; Jankowska, 2017). This finding suggests that some cases of POI diagnosed as idiopathic may include those attributable to insufficient transition of pre-GCs to GCs. In the IVA method, the oocyte-awakening process (PTEN-P3K-akt-Foxo3 signaling) is targeted in order to activate the few remaining primordial follicles. This method is applicable not only to POI patients but also to cancer patients whose only option for having children with their own oocytes is to cryopreserve their ovaries. However, it is hoped that this method will be developed further, because of the 7.8% chance of pregnancy yielded by IVA treatment (Kawamura et al., 2013; Suzuki et al., 2015; Zhai et al., 2016), which is comparable to the estimated 5-10% chance of pregnancy in POI patients (Van Kasteren and Schoemaker, 1999). In this study, treatment of ovarian cultures with the WNT activator CHIR99021 increased the thickness of the GC layer in early developing follicles (oocyte size of 20-40 μm). It is important to note that such a scenario was not observed in response to activation of WNT signaling in Ctnnb1CA mice; however, this difference may be due to a difference in the extent of WNT signaling activation, or to an effect of CHIR99021 on cell survival (Wang et al., 2015). Given that our study demonstrates that a WNT activator induced GC layer thickening and enhanced follicle growth in vitro, transient administration of a WNT activator such as CHIR99021 or WNT proteins on IVG may prove to be clinically beneficial for enhancing the pre-GC transition to GC and thereby to lead efficient PFA and successful pregnancy.

MATERIALS AND METHODS

Animals
Sf1-Cre mice (stock no. 012462), Wlslox/fox mice (stock no. 012888), W1CreERT2 mice (stock no. 010912), Ddx4-Cre mice (stock no. 006954) and Aip mice (stock no. 007909) were obtained from The Jackson Laboratory (Carpenter et al., 2010; Dhillon et al., 2006; Gallardo et al., 2007; Madisen et al., 2010; Zhou et al., 2008). Wlslox/del mice, in which the Wlslox allele is deleted ubiquitously, were generated by crossing Wlslox/fox mice with Ddx4-Cre mice. R26-WntVis mice (accession no. CDB0303 K) were obtained from the Laboratory for Animal Resources and Genetic Engineering at the RIKEN Center for Biosysystems Dynamics Research, Kobe, Japan (http://www.clst.riken.jp/arg/reporter_mice.html) (Takemoto et al., 2016). Ctnnb1lox(ex3) mice were kindly provided by M. M. Taketo (Kyoto University, Japan) (Harada et al., 1999). Sf1-Cre; Wlslox/fox; and Wlslox/del mice were used as littermate controls for Sf1-Cre; Wlslox/fox mice. Tamoxifen-injected W1CreERT2; Wlslox/fox mice and Wlslox/del mice were used as littermate controls for W1CreERT2; Wlslox/fox mice. Wil1lox/+ mice were used for littermate control for W1CreERT2; Ctnnb1lox(ex3) mice. Tamoxifen (0.2 mg per 2 g of body weight) was injected intraperitoneally into mice at P3, P5 and P7. All animal experiments were approved by the Institutional Animal Care and Use Committee of RIKEN (approval number: A2017-13-5). All mouse lines studied were maintained on a mixed genetic background.

Fertility test
Eight-week-old control or Wls cKO female mice (n=7 for each genotype) were housed continuously with WT (C57BL/6N) males for 24 weeks, and the numbers of pups produced were counted.
NM_009089.2, target region 2802-3678), Wnt1 (401091, NM_021279.4, target region 1204-2325), Wnt2 (313601, NM_026563.5, target region 857-2086), Wnt2b (405031, accession no. NM_009520.3, target region 1307-2441), Wnt3 (312241, NM_009652.1, target region 134-1577), Wnt4a (405041, NM_009522.2, target region 667-1634), Wnt5a (316791, NM_009524.3, target region 200-1431), Wnt5b (405051, NM_001271757.1, target region 319-1807), Wnt7a (401121, NM_009527.3, target region 1811-3013), Wnt7b (401131, NM_009528.3, target region 1597-2839), Wnt9a (405061, NM_009290.2, target region 180-1458), and rabbit anti-AMH (1:100; GTX129593, GeneTex), rabbit anti-FOXO3 (1:500; 12829, Cell Signaling Technology), rat anti-MKI67 (1:400; 14-289G, Cell Signaling Technology), and then exposed for 2 h at room temperature to a 1:500 dilution of secondary antibodies labeled with Alexa Fluor 568-conjugated Phalloidin (1:100; A12380, Thermo Fisher Scientific), and subsequently with donkey anti-goat IgG Alexa Fluor 647 (1:500; ab13840, Abcam) and biotinylated secondary antibodies (1:500; BA-1000, Vector Laboratories). Immunocomplexes were detected using a Streptavidin Biotin Complex Peroxidase Kit (30462-30, Nacalai Tesque) and Peroxidase Stain DAB Kit (25985-50, Nacalai Tesque). Nuclei were counterstained with Hematoxylin.

Image analysis
Immunostaining was examined using a BX53 upright microscope (Olympus) or a slide scanner (Axio Scan.Z1, Zeiss). Phalloidin staining was examined using a confocal laser scanning microscope (TCS SP8, Leica Microsystems). In situ hybridization and PAS-H staining were examined using a slide scanner (Axio Scan.Z1, Zeiss). All images taken with Axio Scan.Z1 utilized the tile scan and automated stitching functions. For measurement of Cre recombinination efficiency, ovarian sections of Sfl-Cre;Ai9 or WntCreERT2;Ai9 mice immunostained for FOXL2 were used. Littermate Ai9 mice were used as negative controls. Images were acquired with a slide scanner (Axio Scan.Z1, Zeiss). The presence or absence of tdTomato fluorescence in FOXL2-positive cells was manually identified on randomly selected ovarian sections from three individuals in each group.

For measurement of WntVis or MKI67 signals in pre-GCs/GCs, ovarian sections were subjected to immunofluorescence staining for GFP or MKI67, respectively, as well as for the GC marker FOXL2 and the oocyte marker DDX4. With the use of ImageJ software (National Institutes of Health), areas positive for both FOXL2 and DAPI were determined as nuclear regions of GCs. The fluorescence intensity of GFP or MKI67 in each region was measured. The lower thresholds for GFP- or MKI67-positive cells were set at the value with 99% accuracy in the negative control samples. More than five ovaries for each genotype as well as more than one section per ovary were analyzed. Results were summarized according to follicle type: primordial, intermediate (containing an oocyte surrounded by a mixed single layer of pre-GCs and GCs), primary, and secondary/preantral. Only follicles with a visible nucleolus in the oocyte were analyzed.

For quantitative analysis of follicle numbers, Bouin’s fixed ovarian sections with immunostaining for DDX4 were analyzed. Only follicles with a visible nucleolus in the oocyte were counted. The raw counts of follicle number were multiplied by five to account for the unanalyzed sections and to obtain the estimates of follicle number per ovary. The follicles were classified into primordial follicles (containing an oocyte with a diameter of <20 μm and surrounded by flat pre-GCs), activated primordial follicles (containing an oocyte with a diameter of >20 μm but not containing cuboidal GCs), primary follicles (containing an oocyte surrounded by a single layer of cuboidal GCs), secondary/preantral follicles (containing an oocyte surrounded by two or more layers of GCs), and antral follicles (containing an oocyte surrounded by multilayered GCs with antral cavity). Seven ovaries for each genotype were analyzed.

For quantification of the subcellular localization of FOXO3, images of immunostained ovarian sections for FOXO3 were analyzed using ImageJ. The fluorescence intensity of FOXO3 in cytoplasmic and nuclear
(DAPI-positive) regions of oocytes was measured together with oocyte diameter. The nuclear to cytoplasmic ratio of FOXO3 intensity was then determined. More than five ovaries for each genotype, and more than one section per ovary, were analyzed.

For the quantification of phosho-RPS6, KIT or KITL intensities, images of ovarian sections immunostained for phosho-RPS6, KIT or KITL were analyzed using ImageJ in the oocyte or the GC area. The regions of GC and oocyte were determined manually using ImageJ. More than four ovaries for each genotype, and one section per ovary, were analyzed.

The thickness of the GC layer was determined as half the difference between the diameters of the follicle and the oocyte as measured in PAS-H stained ovarian sections using ImageJ. More than four ovaries for each genotype or treatment, and more than one section per ovary, were analyzed.

For detailed morphometric analyses of growing follicles, primary and secondary follicles were categorized by the appearances of GCs on PAS-H stained Bouin’s fixed sections as follows: squamous: more than 70% of GCs are squamous. The aspect ratio of GCs is approximately 2.0-4.0. Squamous/ cuboidal: a mixture of squamous and cuboidal cells or GCs show the intermediate feature of squamous and cuboidal. Cuboidal: more than 70% of GCs are cuboidal. The cell aspect ratio is approximately 0.8 to 1.2. Cuboidal/ columnar: a mixture of cuboidal and columnar cells or GCs show the intermediate feature of cuboidal and columnar cells. Columnar: nuclei are close to one side of the cytoplasm and show apparent cell polarity. The aspect ratio of GCs is approximately 0.6-0.8. Only follicles with a visible nucleus in the oocyte were counted in randomly selected sections. More than four ovaries for each genotype, and one section per ovary, were analyzed.

Sex genotyping
For genotyping, we used tail or toe (newborn pups only) snips. The genomic DNA was prepared from tails/feet of male and female pups according to the procedures described by Bingham et al. (2004). We used tail or toe (newborn pups only) snips. The genomic DNA was prepared from tails/feet of male and female pups according to the procedures described by Bingham et al. (2004). We used primer sets to detect specific regions to determine the chromosomal sex of each pup. The primer sets used were as follows: male: 5′-TGCTCTGAGGCCAGCACAGCT-3′ and 5′-GCCAGCACGCATCACAATATCCAGATG-3′. The PCR products were separated by 3.0% agarose gel electrophoresis.

Measurement of hormone levels
For measuring hormone levels, the ovaries were removed from 20-day-old mice and immediately placed into a 2-mL tube containing 0.8-ml of phosphate-buffered saline (PBS) with 10% fetal bovine serum. The ovaries were homogenized in a 2-mL tube with a Teflon pestle, and then centrifuged at 14,000 g for 10 minutes. The supernatant was collected and stored at −80°C until analysis. AMH, inhibin A and estradiol concentrations were measured with enzyme-linked immunosorbent assays (Rat and Mouse AMH ELISA, AL-113, Ansh Labs; Equine/Canine/Rodent Estradiol ELISA, M204527200; R&D Systems). Ovaries were treated with 2 µM MCHIR99021 (Sigma-Aldrich) or 100 nM 3BDO (Sigma-Aldrich), or 0.1% DMSO as a vehicle control. The ovaries were maintained at 37°C under 5% O2 and 95% air. Approximately half of the medium in each well was replaced with fresh medium every other day. The ovaries were maintained at 37°C under 5% CO2 and 95% air.

Statistical analysis
All statistical analysis was performed using GraphPad Prism 8 or 9 software. Tests included the nonparametric Mann–Whitney matched-pairs test, unpaired multiple t-tests with the Holm-Sidak correction, two-way ANOVA with Sidak’s post hoc test for multiple comparisons, and a chi-squared test for trend for the contingency table. A P-value of <0.05 was considered statistically significant.

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Competing interests
The authors declare no competing or financial interests.

Author Contributions
Conceptualization: H.M.T.; Investigation: O.H., C.Y.L., H.M.T.; Writing - original draft: H.M.T.; Writing - review & editing: O.H., C.Y.L., M.K.-A., R.N.; Supervision: M.K.-A., R.N.; Project administration: H.M.T.; Funding acquisition: H.M.T., R.N.

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References
Adhikari, D. and Liu, K. (2009). Molecular mechanisms underlying the activation of mammalian primordial follicles. Endocr. Rev. 30, 438-464. doi:10.1210/er.2008-0048
Adhikari, D., Zheng, W., Shen, Y., Gorre, N., Hämäläinen, T., Cooney, A. J., Huhtanenmi, I., Lan, Z. J. and Liu, K. (2009). Tsc/mTORC1 signaling in oocytes governs the quiescence and activation of primordial follicles. Hum. Mol. Genet. 19, 397-410. doi:10.1093/hmg/ddp483
Albertini, D. F., Combelles, C. M. H., Benecchi, E. and Carabatos, M. J. (2001). Cellular basis for paracrine regulation of ovarian follicle development. Reproduction 121, 647-653. doi:10.1530/reprod.1.201647
Bingham, N. C., Verma-Kurvari, S., Parada, L. F. and Parker, K. L. (2002). Development of a steroidogenic factor 1/Cre transgenic mouse line. Genesis 44, 419-424. doi:10.1002/dvg.20231
Boerboom, D., Paquet, M., Hsieh, M., Liu, J., Jamin, S. P., Behringer, R. R., Siros, J., Taketo, M. M. and Richards, J. A. S. (2005). Misregulated Wnt3a/catenin signaling to ovarian granulosa cell tumor development. Cancer Res. 65, 9206-9215. doi:10.1158/0008-5472.CAN-05-1024
Boerboom, D., White, L. D., Dalle, S., Courtj, Y. and Richards, J. A. S. (2006). Dominant-stable β-catenin expression causes cell fate alterations and Wnt signaling antagonist expression in a murine granulosa cell tumor model. Cancer Res. 66, 1964-1973. doi:10.1158/0008-5472.CAN-05-3493
Boyer, A., Lapointe, E., Zheng, X., Cowan, R. G., Li, H., Quirk, S. M., Demayo, F. J., Richards, J. S. and Boerboom, D. (2010). WNT4 is required for normal ovarian follicle development and female fertility. FASEB J. 24, 3010-3025. doi:10.1096/fj.09-145799
Carabatos, M. J., Elvin, J., Matzuk, M. M. and Albertini, D. F. (1998). Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. Dev. Biol. 204, 375-384. doi:10.1006/dbio.1998.9087
Carpenter, A. C., Rao, S., Wells, J. M., Campbell, K. and Lang, R. A. (2010). Generation of mice with a conditional null allele for Wnt4. Genesis 58, 545-555. doi:10.1002/dvg.200651
Castrillon, D. H., Miao, L., Kollipara, R., Horner, J. W. and DePinho, R. A. (2003). Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science (80-.) 301, 215-218. doi:10.1126/science.1096336
Chassot, A. A., Gillot, I. and Chaboissier, M. C. (2014). R-Spindlin1, WNT4, and the ctnnb1 signaling pathway: Strict control over ovarian differentiation. Reproduction 148, R97-110. doi:10.1530/REP-14-0177
Chen, B., Dodge, M. E., Tang, W., Lu, J., Ma, Z., Fan, C. W., Wei, S., Hao, W., Kilgore, J., Williams, N. S. et al. (2009). Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. Nat. Chem. Biol. 5, 100-107. doi:10.1038/nchembio.137
De Cian, M. C., Pauper, E., Bandiera, R., Vidal, V. P. I., Sacco, S., Gregoire, E. P., Le Rolle, M., Lachambre, S., Mondin, M., Bell, S., Chassot, A. A., Panzolini, C., Wilhelm, D., Pailhoux, E. et al. (2021). Amplification of R-spondin1 signaling induces granulosa cell fate defects and ovariole disruption and Akt stimulation of ovarian follicles for infertility treatment. J. Cell Mol. Med. 25, 4065-4075. doi:10.1177/15307656211017040
following ovarian tissue vitrification in patients with primary ovarian insufficiency. 

Hum. Reprod. 30, 608-615. doi:10.1093/humrep/deu353

Takemoto, T., Abe, T., Kiyonari, H., Nakao, K., Furuta, Y., Suzuki, H., Takada, S., Fujimori, T. and Kondo, H. (2016). R26-WntVis reporter mice showing graded response to Wnt signal levels. Genes Cells 21, 661-669. doi:10.1111/gtc.12364

Vainio, S., Heikkila, M., Kispert, A., Chin, N. and McMahon, A. P. (1999). Female development in mammals is regulated by Wnt-4 signalling. Nature 397, 405-409. doi:10.1038/17066

Van Kasteren, Y. M. and Schoemaker, J. (1999). Premature ovarian failure: a systematic review on therapeutic interventions to restore ovarian function and achieve pregnancy. Hum. Reprod. Update 5, 483-492. doi:10.1093/humupd/5.5.483

Visser, J. A., de Jong, F. H., Laven, J. S. E. and Themmen, A. P. N. (2006). Anti-Müllerian hormone: A new marker for ovarian function. Reproduction 131, 1-9. doi:10.1530/rep.1.00529

Wang, F., Flanagan, J., Su, N., Wang, L. C., Bui, S., Nielson, A., Wu, X., Vo, H. T., Ma, X. J. and Luo, Y. (2012). RNAseq: A novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J. Mol. Diagnostics 14, 22-29. doi:10.1016/j.jmoldx.2011.08.002

Wang, X., Wei, L., Cramer, J. M., Leibowitz, B. J., Judge, C., Epperly, M., Greenberger, J., Wang, F., Li, L., Stelzner, M. G. et al. (2015). Pharmacologically blocking p53-dependent apoptosis protects intestinal stem cells and mice from radiation. Sci. Rep. 5, 8566. doi:10.1038/srep08566

Wiese, K. E., Nusse, R. and van Amerongen, R. (2018). Wnt signalling: Conquering complexity. Development 145, dev165902. doi:10.1242/dev.165902

Yamashiro, C., Sasaki, K., Yabuta, Y., Kojima, Y., Nakamura, T., Okamoto, I., Yokobayashi, S., Murase, Y., Ishikura, Y., Shirane, K. et al. (2018). Generation of human oogonia from induced pluripotent stem cells in vitro. Science (80-) 362, 356-360. doi:10.1126/science.aat1674

Yu, J., Yaba, A., Kasiman, C., Thomson, T. and Johnson, J. (2011). mTOR Controls ovarian follicle growth by regulating granulosa cell proliferation. PLoS One 6, e21415. doi:10.1371/journal.pone.0021415

Zhai, J., Yao, G., Dong, F., Bu, Z., Cheng, Y., Sato, Y., Hu, L., Zhang, Y., Wang, J., Dai, S. et al. (2016). In Vitro activation of follicles and fresh tissue autotransplantation in primary ovarian insufficiency patients. J. Clin. Endocrinol. Metab. 101, 4405-4412. doi:10.1210/jc.2016-1589

Zhou, B., Ma, Q., Rajagopal, S., Wu, S. M., Domian, I., Rivera-Feliciano, J., Jiang, D., Von Gise, A., Ikeda, S., Chien, K. R. et al. (2008). Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. Nature 454, 109-113. doi:10.1038/nature07060