WNT/β-catenin and p27/FOXL2 differentially regulate supporting cell proliferation in the developing ovary

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1. Introduction

Formation of a testis or ovary initiates male and female development and the establishment of the spermatogenic and oogenic programs in mammals. In mice, gonad development begins through formation of a bipotential genital ridge that is characterised by a thickening of the coelomic epithelium, a process that is dependent on several transcription factors including GATA4 (GATA binding protein 4) (Hu et al., 2013). Following formation of the genital ridge, pre-supporting cells in XX and XY individuals undergo specification to the granulosa and Sertoli cell lineages, respectively. These lineages are derived from cells that express GATA4 in both XX and XY individuals and GATA4 is required for both ovary and testis development (Anttonen et al., 2003; Manuylov et al., 2008; Teyssen et al., 2002). While GATA4 functions with its co-factor FOG2 (friend of GATA) in early gonad development and during sex differentiation, GATA4 regulates ovarian follicle formation independently of FOG2 (Efimenko et al., 2013).

In addition to GATA4, ovarian development requires female-specific functions mediated by Wnt4 (Wingless-related MMTV integration site 4), Rspo1 (R-spondin homologue), Ctnnb1 (β-catenin) and Foxl2 (Forkhead box L2), which establish fetal granulosa cells and regulate folliculogenesis (Chassot et al., 2008; Garcia-Ortiz et al., 2009; Kim et al., 2006; Maatouk et al., 2008; Ortiz et al., 2009; Kim et al., 2006).

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**ABSTRACT**

Sexual development is initiated through differentiation of testicular Sertoli cells or ovarian granulosa cells. Although these supporting cells are considered to develop from common bipotential precursors, recent evidence suggests that distinct supporting cell populations are present in the ovary, with one providing granulosa cells of the medullary follicles and the other providing granulosa cells of the cortical follicles, the latter of which support lifelong fertility. Here, we demonstrate that XX fetal gonads contain GATA4 expressing supporting cells that either enter mitotic arrest, or remain proliferative. Blocking WNT signalling reduces XX supporting cell proliferation, while stabilising β-catenin signalling promotes proliferation, indicating that the renewal of pre-granulosa cells is dependent on WNT/β-catenin signalling in the proliferative supporting cell population. In contrast, XX supporting cells express p27 and FOXL2 and are maintained in mitotic arrest. Although FOXL2 is required for maintaining high levels of p27 expression, it is dispensable for entry and maintenance of mitotic arrest in XX supporting cells. Combined our data suggest that both medullary and cortical precursors arise from a common GATA4 expressing cell type. In addition, this work indicates that a balance between supporting cell self-renewal and differentiation is maintained in the developing ovary by relative WNT/β-catenin and p27/FOXL2 activities. This study provides significant new insights into the origin and formation of ovarian follicles and evidence supporting a common fetal origin of medullary and cortical granulosa cells.

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Ottolenghi et al., 2007; Tomizuka et al., 2008; Vainio et al., 1999; Yao et al., 2004). In humans, heterozygous mutations in FOXL2 lead to blepharophimosis-ptosis-epicanthus-inversus syndrome and premature ovarian failure, but not sex-reversal (Crisponi et al., 2001). The consequences of homozygous FOXL2 mutations in humans remain unknown, but homozygous loss of FOXL2 in goats results in female to male sex reversal (Boulanger et al., 2014). Although morphologically normal fetal ovaries develop in Foxl2 null mice (Schmidt et al., 2004; Uda et al., 2004) genes regulating testis development are upregulated shortly before birth (Garcia-Ortiz et al., 2009) and follicle activation is impaired in post-natal stages (Schmidt et al., 2004; Uda et al., 2004).

For many years it has been thought that fetal granulosa and Sertoli cells are derived from a bipotential precursor cell that is present in the bipotential gonads of XX and XY individuals, respectively. In the presence of SRY either XX or XY pre-supporting cells can establish Sertoli cells, while loss of SRY function leads to granulosa cell commitment (Lin and Capel, 2015). However, recent findings suggest that this paradigm is more complex in the XX gonad (Mork et al., 2012; Rastetter et al., 2014; Zheng et al., 2014). At the earliest stages of ovarian development a population of non-proliferative somatic cells express FOXL2 and the cell cycle inhibitor p27KIP1 (p27), and establish medullary follicles (Mork et al., 2012; Rastetter et al., 2014) that are activated from birth to post-natal day (PND) 45, before declining to around 2.4% of the follicle pool by PND90 (Hummitzsch et al., 2015; Zheng et al., 2014). A second population of pre-granulosa cells arises from proliferative epithelial cells during E15.5 to PND4 and establishes cortical follicles, which contribute to the life-long follicular reserve. However, although cortical and medullary granulosa cells arise at different developmental stages, it remains possible that they are derived from a common progenitor pool (Mork et al., 2012).

In conjunction with Foxl2, ovarian development also requires the canonical WNT4/RSPO1/β-catenin signalling pathway. Loss of function mutations in WNT4 or RSPO1 underlie male to female sex reversal in humans (Mandel et al., 2008; Parma et al., 2006; Radi et al., 2005; Tomaselli et al., 2008). In mice, loss of WNT4/β-catenin signalling results in development of the testis-specific coelomic vessel, de-repression of male steroidogenic enzymes and disrupted germ cell development, but does not result in formation of testes from the bipotential gonad in XX individuals (Chassot et al., 2008; Tomizuka et al., 2008; Vainio et al., 1999). Despite this, β-catenin and WNT4 antagonise the pro-testis genes Sox9 (SRy-box containing gene 9) and Fgf9 (fibroblast growth factor 9), while testis-like cords form in gonads of late stage Wnt4 or Rspos1 null XX fetuses (Chassot et al., 2008; Kim et al., 2006; Maatouk et al., 2008; Vainio et al., 1999), demonstrating a role for WNT4/β-catenin in repressing male pathways. WNT4 and RSPO1 also play a number of roles in promoting ovarian development through the β-catenin pathway.

Precursors of cortical granulosa cells are marked by WNT4 and RSPO1 dependent expression of the RSPO receptor LGR5 (Rastetter et al., 2014), but it remains unknown whether LGR5 is required for follicle formation as Lgr5-null mice die perinatally (Morita et al., 2004). However, a recent study demonstrates that the related gene Lgr4 is required for full WNT/β-catenin signalling in the developing ovary. Lgr4 loss of function mutants contained fewer FOXL2 positive supporting cells and were partially masculinised (Kozumi et al., 2015). In addition, deleting WNT4 initially involves the loss of p27 and premature activation of follicles prior to formation of testis-like cords (Maatouk et al., 2013). In this context, the loss of p27 appears to be an indirect consequence of altered WNT4/β-catenin signalling as depletion of germ cells rescues mitotic arrest of the supporting cell population (Maatouk et al., 2013). Moreover, loss of p27 also leads to premature activation of follicles and early infertility, which involves early oocyte growth but not granulosa cell hyperproliferation (Rajareddy et al., 2007). Foxl2 expression is also partly dependent on RSPO1, β-catenin and Wnt4, while deletion of both Wnt4 and Foxl2, or Rspos1 and Foxl2, results in a more severe phenotype than the respective single knockout models. Gonads in these mice develop as ovotestes rather than testes, indicating partial sex-reversal (Auguste et al., 2011; Chassot et al., 2014; Ottolenghi et al., 2007).

Here, we have developed flow cytometric assays to dissect the early stages of ovarian development. We show that the undifferentiated gonad contains mitotically arrested GATA4+ve somatic cells that express p27 at the time FOXL2 expression is initiated in the XX bipotential gonad. In addition, the developing ovary contains GATA4+ve cells that lack both p27 and FOXL2, and rapidly proliferate. These proliferative GATA4+ve cells express LGR5, an RSPO1 receptor that facilitates WNT signalling, supporting a role for WNT/β-catenin in cell proliferation in the developing ovary. Consistent with this, we show that blocking the β-catenin antagonist glycogen synthase kinase 3 (GSK3) promotes proliferation, while blocking WNT signalling reduces proliferation of GATA expressing supporting cells. Combined, these data demonstrate that WNT/β-catenin signalling promotes proliferation of supporting cells in the developing ovary, while FOXL2 functions in a mitotically arrested population of GATA4+ve supporting cells that contribute to both medullary and cortical follicles. Based on these and published data, we provide a model for supporting cell recruitment in XX gonads outlining early events underpinning ovary formation.

2. Materials and methods

2.1. Animals

Mice used in all experiments were derived from: OG2 (Oct4-GFP; octamer-binding transcription factor 4, also known as Pou5f1) transgenic male (pure 129T2SvJ) (Szabo et al., 2002) x Swiss female matings; Foxl2+/− x Foxl2+/− (Ottolenghi et al., 2005); Swiss x Swiss matings; and Lgr5-GFP+/− x Lgr5-GFP+/− (Rastetter et al., 2014). The presence of a vaginal plug in the morning was used to indicate mating and was recorded as E0.5. Fetuses were staged as described (Western et al., 2011) and by regularly counting tail somites (ts) in representative E11.5 and E12.5 fetuses (E11.5 approx. 17–20 ts; E12.5 approx. 26–29 ts). All animal procedures were carried out under Monash University Animal Ethics Committee and Murdoch Childrens Research Institute Animal Ethics Committee approvals.

2.2. Fluorescent activated cell sorted somatic and germ cell RNA extraction and qRT-PCR

Germ and somatic cells were sorted from Oct4-GFP embryonic gonads and RNA extracted, and amplified as described (van Bergen et al., 2009; Western et al., 2008). The purity of this material has been extensively verified using independent assays (van Bergen et al., 2009; Western et al., 2008; Western et al., 2010). PCR was performed on an ABI7900 (Life Technologies) instrument using UPL probes (Roche) to monitor product levels. Details of primer sets and UPL Probes used are shown in Supplementary Table 1. Results were analysed using the manufacturers’ software. Relative expression levels were determined by normalisation against verified reference genes Sdha and Mapk1 (van den Bergen et al., 2009). Data is representative of biological triplicate samples, with PCRs performed in technical duplicate.
2.3. Organ culture

E11.5 gonad/mesonephros or E12.5 gonad only samples were collected from Oct4-GFP transgenic fetuses and sexed using PCR as previously described (McFarlane et al., 2013; Miles et al., 2013). XX or XY gonad/mesonephros or gonad tissues were placed onto 30 mm Millicell Biopore 0.4 μm membranes (Millipore) in six-well plates containing 1200 μl organ culture media (250 μM sodium pyruvate, 15 mM Hepes, 1X non-essential amino acids, 1 mg/ml N-acetylcysteine, 55 μM β-mercaptoethanol and 10% FCS in DMEM/F12 with Glutamax). Chir99052 (GSKi; 3 μM, Cayman Chemicals) (Bennett et al., 2002; Ying et al., 2008) or IWR1 (WNTi; N-acetylcysteine, 55 μM) were added to the culture medium at the outset of the experiment. Tissues were cultured for 24 h or 72 h in 5.0% CO2 at 37 °C. Cultured gonads were dissected at the final 2 h of culture. Gonads were photographed under bright field and fluorescence optics, removed from filters and either dissociated using trypsin and fixed for flow cytometric analysis (Wakeling et al., 2013), fixed whole for immunofluorescence in 4% paraformaldehyde (PFA).

2.4. Flow cytometry

Antibody staining and flow cytometry were performed as described (Wakeling et al., 2013), with the exception that 1–2 gonads were analysed in each sample and antibodies specific to this study were used (Supplementary Table 2). Pregnant mothers were injected intraperitoneally (i.p) with 20 mg/kg EdU to facilitate in vivo analysis of gonadal cell proliferation. Two hours after the i.p injection the mothers were sacrificed, fetuses removed, gonads dissected, dissociated and the cells fixed for flow cytometry. For analyses of cell proliferation in cultured gonads, EdU was added at a final concentration of 20 μM to the organ culture media for the final 2 h of culture after which gonads were collected, dissociated and the cells fixed for flow cytometry. Initially, antibodies were titrated in the appropriate target cell population to optimise staining concentration. Tissues that did not express the protein of interest were used as negative controls to set flow cytometry gates (Supp. Fig. 1 and Supp. Fig. 2). For example, XY gonads were used as controls for FOXL2, XX gonads were used as controls for SOX9 and AMH (Supp. Fig. 1) and mesonephros was used as a control for GATA4 and p27 staining (Supp. Fig. 2). Where FOXL2/p27 antibodies were combined (both raised in rabbit), Zenon Labelling (Life Technologies) was used to directly label the p27 antibody (Miles et al., 2013). Since the EdU staining process destroys GFP fluorescence, we used antibodies specific for the germ cell marker Mouse Vasa Homologue (MVH) in cases where EdU staining was performed (Hogg and Western, 2015). Unless otherwise stated in the figure legend, 3–10 biologically distinct samples were analysed and statistical significance was determined using ANOVA with Tukey’s multiple comparison test for experimental groups > 2, or unpaired t-test. A p-value of less than 0.05 was considered significant.

2.5. Tissue fixation and immunofluorescence

Cultured gonads were fixed in PBS containing 4% PFA for 20–45 min (depending on gestational age) at room temperature and mounted in optimal cutting temperature (OCT) compound. Cryosections were cut at 10 μm and immunofluorescence (IF) was performed as previously described (Miles et al., 2010; Rastetter et al., 2014; Western et al., 2008; Western et al., 2011). Details of primary and secondary antibodies are provided in Supplementary Table 2.

3. Results

3.1. FOXL2/p27 expressing cells are mitotically arrested in the fetal ovary

Differential proliferation is a prominent sexually dimorphic characteristic of somatic cell development in the XX and XY gonads (Mork et al., 2012; Schmahl et al., 2000). Initially we developed flow cytometric approaches to quantify supporting cell proliferation in the fetal ovary. Two hours prior to collection of fetuses, pregnant female mice were exposed to EdU, which is incorporated during DNA synthesis, thereby marking cells progressing through S-phase of the cell cycle. We dissected gonads and analysed supporting cell proliferation using antibodies specific to FOXL2 in XX gonadal cells and SOX9 in XY gonadal cells, combined with EdU and propidium iodide staining, enabling assessment of DNA content and cell cycle stage in each cell. XY gonads were used as negative controls for FOXL2 staining, while XX gonads were used as negative controls for SOX9 staining (Supp. Fig. 1A and B).

Less than 1% of FOXL2 expressing cells in XX gonads incorporated EdU and contained a 2n DNA content demonstrating that these cells were arrested in G0/G1 of the cell cycle at E12.5, E13.5 and E14.5. By contrast, 19.5% of the SOX9 expressing cells in E12.5 XY gonads incorporated EdU demonstrating that they were highly proliferative (Supp. Fig. 2A). Consistent with the mitotically arrested state of FOXL2 expressing cells (Mork et al., 2012), double IF in tissue sections of E12.5 XX gonads demonstrated that FOXL2 co-localised with the cell cycle inhibitor p27 (Supp. Fig. 2B).

In addition, assessment of mRNA expression in E12.5–E15.5 XX and XY somatic cells purified by Oct4-GFP dependent fluorescence activated cell sorting (FACS) (van den Bergen et al., 2009; Western et al., 2008) showed that the cell cycle inhibitors p27kip1, p21cip1, p57kip2 were robustly expressed in XX, but not XY somatic cells. However, p15ink4b (Cdkn2b) and p16ink4a (Cdkn2a) were detected at minimal levels in both XX and XY somatic cells (Supp. Fig. 2C). The gene encoding the G1-S phase check-point protein, p27kip1, was expressed at moderate levels in somatic cells (Supp. Fig. 2C). Combined these data corroborate previous observations that ovarian somatic cells express FOXL2 and G1-S inhibitors, are not proliferating (Cederroth et al., 2007; Mork et al., 2012; Nef et al., 2005).

3.2. GATA4 marks both mitotically arrested and proliferative cells in the XX fetal gonad

Somatic cells in the developing ovary are known to express GATA4. To quantitatively determine the constitution of E12.5 XX gonads we developed flow cytometric assays using antibodies specific for GATA4, p27 and FOXL2, while separating germ cells using Oct4-GFP or MVH staining (Supp. Fig. 3). Initially, combinations of triple labelling for p27/GATA4/GFP, FOXL2/GATA4/GFP, p27/FOXL2/GFP were performed. To complement this we analysed combinations of p27/GATA4/EdU and GATA4/FOXL2/EdU, each in combination with propidium iodide, to reveal the relative DNA content per cell. These combinations allow analyses of proliferation and cell cycle state in the respective GATA4/FOXL2, GATA4/p27 and FOXL2/p27 positive and negative somatic cell populations. Combined, these analyses demonstrated that E12.5 XX gonads contained 81% somatic cells and 19% germ cells (Fig. 1A i). The somatic population contained GATA4+/ve cells (82%) and GATA4–/ve cells (18%) (Fig. 1A ii). Of the GATA4+/ve cells, 69% were p27+/ve and 31% were p27–/ve (Fig. 1A iii). Almost all (99%) of the p27–/ve cells in the XX gonad expressed GATA4, were in G0/G1 of the cell cycle and did not incorporate EdU, demonstrating that they were in mitotic arrest (Fig. 1B i). In contrast, 28% of the GATA4+/ve/p27–/ve cells incorporated EdU, demonstrating that they
were actively engaged in S-phase and highly proliferative (Fig. 1B ii). We next determined the distribution of FOXL2 in the GATA4 and p27 populations by staining for GATA4 and FOXL2, as well as p27 and FOXL2 and separating germ cells using GFP. This revealed that while 99% of the FOXL2 $^+$ve cells expressed GATA4 only 33% of the GATA $^+$ve cells expressed FOXL2 (Fig. 1C). Consistent with this, analysis of the p27 $^+$ve cell population in E12.5 XX gonads revealed that 63% expressed FOXL2, demonstrating that not all p27 $^+$ve cells in the E12.5 XX gonad express FOXL2 (Fig. 1D). Since 99% of the GATA4/p27 $^+$ve cells were in mitotic arrest at E12.5 (Fig. 1B i) and only 63% expressed FOXL2, this also demonstrates that FOXL2 expression is unlikely to initiate mitotic arrest in these cells. Conversely, since 99% of the FOXL2 $^+$ve population failed to incorporate EdU (Supp. Fig. 3A), essentially all FOXL2 expressing cells were in mitotic arrest at E12.5. These data indicate that in the E12.5 XX gonad, around one third of the GATA4 $^+$ve cells are p27 $^-ve$ and highly proliferative, while the remaining GATA4 $^+$ve cells are p27 $^+$ve and are in mitotic arrest. Moreover, while approximately two thirds of the p27 $^+$ve cells express FOXL2 in the E12.5 XX gonad, a substantial population of p27 $^+$ve expressing cells are present that do not express FOXL2 but are already mitotically arrested.

3.3. GATA4 expressing pre-supporting cells contribute to the pre-granulosa and pre-Sertoli cells of the fetal ovary and tests

We next analysed the onset of mitotic arrest and supporting cell differentiation in the developing XX gonad using flow cytometry. To determine whether the bipotential gonad contains somatic precursors that are p27 $^+$ve we performed flow analyses of gonad/mesonephric tissues isolated from E11.5 XX and XY fetuses. E11.5 XX and XY gonads contained equivalent proportions of MVH $^+$ve germ cells (Supp. Fig. 4A i), GATA4 $^+$ve somatic cells (Supp. Fig. 4A ii), and p27 $^+$ve cells (Supp. Fig. 4A iii). Approximately 90% of the p27 $^+$ve cells expressed GATA4 in both sexes (Supp. Fig. 4A iv). These p27 $^+$ve cells were located in G$_0$/G$_1$ of the cell cycle (2n) at E11.5 (Fig. 2A i), consistent with their arrest in G$_0$/G$_1$ at E12.5 (Fig. 1B and Fig. 2A ii). Importantly, flow cytometric analysis of separated E12.5 gonad and mesonephric tissues demonstrated that >99% of the p27 $^+$ve cells were found in the gonad (Supp. Fig. 5A). Similarly, IF analysis of E11.5 gonad/mesonephric tissues also revealed p27 $^+$ve cells in the gonad, but not in the mesonephros (Supp. Fig. 5B). Combined, these data strongly indicate that all p27 $^+$ve cells detected by flow analysis in E11.5 gonad/mesonephric tissues originate from the gonad.
Next, we used flow cytometry in XX and XY E11.5 gonad/mesonephros tissues to determine the proportion of pre-supporting cells committing to the granulosa and Sertoli cell lineages and whether these cells express GATA4 and p27. At E11.5 a subset of XX and XY GATA4\(^+\)ve cells expressed FOXL2 or SOX9, respectively (Supp. Fig. 4B). Proportionally half as many E11.5 GATA4\(^+\)ve cells expressed FOXL2 in the XX gonad compared to GATA4\(^+\)ve cells expressing SOX9 in the XY gonad (Supp. Fig. 4B iii). In both cases FOXL2 and SOX9 expression was initiated in cells that also expressed p27. At E11.5, 17% and 51% of the XX and XY p27\(^+\)ve cells also expressed FOXL2 and SOX9, respectively (Fig. 2A, Supp. Fig. 4C). Although only 17% of the p27\(^+\)ve XX somatic cells were FOXL2\(^+\)ve at E11.5, this increased to 63% and 80% by E12.5 and E13.5, respectively (Fig. 2A iii). Since most (99%) FOXL2\(^+\)ve cells were in mitotic arrest at E12.5 and E13.5 (Fig. 1A; Mork et al., 2012), these data demonstrate that p27\(^+\)ve cells begin to express FOXL2 between E11.5 and E13.5 rather than accumulating through proliferation.

Interestingly, only 61% of the FOXL2\(^+\)ve cells expressed p27 at E11.5, but this increased to 87% and 90% at E12.5 and E13.5, respectively, indicating that p27 expression may also be activated in FOXL2 positive cells during this period (Fig. 2B). However, a caveat is that as both the p27 and FOXL2 antibodies were raised in rabbit, the p27 antibody was directly labelled using Zenon Alexa647 in these assays. As Zenon labelled p27 is marginally less sensitive in flow cytometry than the p27 antibody detected using secondary antibodies, this may lead to a slight under-estimation of the proportion of p27\(^+\)ve cells using the Zenon labelling assay. Therefore, this may account for the slightly inconsistent observations that p27 was detected in \(\sim 90\%\) of FOXL2\(^+\)ve cells at E12.5 and E13.5, but 99% of FOXL2\(^+\)ve cells are mitotically arrested at these stages. However, this is unlikely to explain the low proportion of p27\(^+\)ve cells detected in the FOXL2\(^+\)ve population at E11.5. We cannot discount a role for p21 or p57 in the arrest of these cells (Supp. Fig. 2C), and these cell cycle regulators may explain the mitotically arrested state of the FOXL2\(^+\)ve cells in which p27 was not detected.

In the XY gonad/mesonephros, 86% of the SOX9\(^+\)ve cells in E11.5 XY gonads expressed p27 at E11.5. However, in contrast to the FOXL2\(^+\)ve cells in the XX gonad, which remained p27\(^+\)ve, all pre-Sertoli cells in the XY gonad had become p27\(^-/\)ve by E12.5 and remained p27\(^-/\)ve at E13.5 (Fig. 2C). While the p27 antibody was Zenon labelled in the SOX9/p27 assays for the E11.5 samples, use of a goat anti-AMH antibody prevented the requirement for Zenon labelling the p27 antibody in the E12.5 and E13.5 assays. Specificity of the AMH antibody was validated using XX gonadal controls.

**Fig. 2.** FOXL2 expression is induced in p27\(^+\)ve supporting cells of the XX gonad while SOX9 expression is followed by repression of p27 in XY gonads. A. Flow cytometric analysis in XX samples: (i). p27, and FOXL2 in the p27\(^+\)ve population, of E11.5 gonad/mesonephros; (ii). p27, and FOXL2 in the p27\(^+\)ve population, of E12.5 gonads, and; (iii). Relative proportions of FOXL2\(^+\)ve cells in the p27\(^+\)ve population of E11.5–E13.5 XX gonads. B. Flow cytometric analysis in XX samples: (i). FOXL2, and p27 in the FOXL2\(^+\)ve population, of E11.5 gonad/mesonephros; (ii). FOXL2, and p27 in the FOXL2\(^+\)ve population, of E12.5 gonads, and; (iii). Relative proportions of p27\(^+\)ve cells in the FOXL2\(^+\)ve population of E11.5–E13.5 gonads. C. Flow cytometric analysis in XY samples: (i). SOX9, and p27 in the SOX9\(^+\)ve population, of E11.5 gonad/mesonephros; (ii). AMH, and p27 in the AMH\(^+\)ve population, and; (iii). Relative proportions of SOX9\(^+\)ve (E11.5) and AMH\(^+\)ve (E12.5–E13.5) cells in the p27\(^+\)ve population of E11.5–E13.5 gonads. Statistics are ANOVA with Tukey’s multiple comparison test where a, b and c are significantly different (\(p < 0.05\)) and values are mean ± SEM (n ≥ 3).
and by double staining with the SOX9 antibody in XY samples (Supp. Fig. 1D).

In summary, these data demonstrate that: (1) E11.5 XX and XY gonads contain equivalent populations of p27+/ve somatic cells that are considerably more common than either FOXL2 or SOX9 expressing cells; (2) FOXL2 expression is up-regulated in the p27+/ve population in the XX gonad, but not all FOXL2+/ve cells are initially p27+/ve, indicating that p27 may also be activated after FOXL2 in some cells; (3) SOX9 expression is up-regulated in the p27+/ve population in the E11.5 XY gonad, and; (4) FOXL2+/ve cells maintain p27 expression in the supporting cells of the E12.5-E13.5 XX gonad and remain in mitotic arrest, while all SOX9+/ve cells repress p27 and are rapidly proliferating in the E12.5 XY gonad.

3.4. FOXL2 is required for the maintenance of p27 in XX somatic cells, but mitotic arrest is not disrupted in Foxl2 null mice

Previous reports have indicated that p27kip1 is transcriptionally reduced in XX gonads of Foxl2-null mice (García-Ortiz et al., 2009). To determine whether FOXL2 is required to maintain p27 levels and mitotic arrest in the somatic cells of XX gonads we analysed Foxl2 wild type (Foxl2+/+), heterozygous (Foxl2+-/) and homozygous null (Foxl2--/) mice (Ottolenghi et al., 2007). Flow cytometric analyses demonstrated a 2-fold reduction in p27 levels in the GATA4+/ve/p27+/ve somatic cells of E15.5 XX gonads of Foxl2--/-- compared to Foxl2+/+ and Foxl2+/-- mice, although the proportion of p27+/ve cells was unchanged (Fig. 3A). Consistent with this, a substantial reduction in p27 levels was observed in somatic cells using IF in E11.5 and E15.5 XX gonads of Foxl2--/-- compared to Foxl2+/+ mice (Fig. 3B; E13.5 only shown). Although this revealed a role for FOXL2 in maintaining p27 in XX somatic cells, flow analyses demonstrated no difference in proliferation of the p27+/ve cell population (Fig. 3C i, ii) or the GATA4+/ve/p27--/ve cells (Fig. 3C iii) in XX gonads of E15.5 Foxl2+/+ Foxl2+/-- and Foxl2--/-- fetuses. In addition, there were no differences in the numbers of germ cells, their expression of MVH, their entry into meiosis at G2/M of the cell cycle (Fig. 3D), or phosphorylated H2AX (γH2AX), a key marker of meiosis (Fig. 3D).

3.5. The granulosa progenitor cell marker LGR5 is expressed in GATA4+/ve cells that contribute to the p27+/ve and p27--/ve populations of the developing ovary

GATA4/p27 staining demonstrates the presence of two GATA4+/ve cell populations (mitotically arrested GATA4+/p27+/ve and proliferative GATA4+/ve/p27--/ve cells) and a GATA4--/ve population in the developing ovary. LGR5 is a marker of proliferative cells that are considered to be granulosa progenitor cells and contribute to cortical follicles in the late fetal and neonatal ovary (Ng et al.,

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**Fig. 3.** Foxl2 maintains p27 expression in GATA4+/ve cells of the developing ovary but is not required for maintaining mitotic arrest in XX somatic cells. A. Flow cytometric analysis of p27 intensity and the proportions of p27+/ve cells in GATA4+/ve cells of E15.5 Foxl2+/+, Foxl2+-/+ and Foxl2--/-- gonads. B. Immunofluorescence analysis of FOXL2 (green) and p27 (red) staining in E13.5 and E15.5 Foxl2+/+ and Foxl2--/-- gonads. Scale bars: 100 μm (top images) and 20 μm (bottom images). M denotes the mesonephric side of the gonad. C-D. Flow analysis of E13.5 Foxl2+/+, Foxl2+-/+ and Foxl2--/-- XX gonads: C. (i) p27 expression in GATA4+/ve cells; (ii-iii). Cell cycle state in GATA4+/ve and GATA4/p27--/ve cells. D. i. MVH+/ve germ cells (MVH+/ve and γH2AX+/ve germ cells); (ii). Cell cycle state in MVH+/ve and (iii). MVH+/γH2AX+/ve germ cells. Statistics are ANOVA with Tukey’s multiple comparison test where a and b are significantly different (p < 0.05) and values are mean ± SEM (n ≥ 3). No significant changes were observed in C and D. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
To determine whether LGR5 exclusively marks proliferating p27/C0ve/GATA4þve cells we examined localisation of GATA4, p27 and Lgr5-GFP using IF in ovaries of E13.5 and E16.5 Lgr5-GFP fetuses (Rastetter et al., 2014). At E13.5, although Lgr5-GFP was detected in many GATA4þve cells that were p27–ve, GATA4þve cells positive for both Lgr5-GFP and p27 were also detected (Fig. 4A). Moreover, these GATA4+/p27+/Lgr5-GFP+/ve cells appeared to be populating the medulla of the fetal ovary, indicating that they were contributing to medullary follicles (Fig. 4A). Although some GATA4+/p27+/Lgr5-GFP+/ve cells were also detected in E16.5 ovaries, there was a distinct demarcation between GATA4+/p27–ve/Lgr5-GFP+/ve cells located in the cortex and the GATA4+/p27+/Lgr5-GFP–ve cells located in the medulla (Fig. 4B). These data are consistent with GATA4+/ve cells establishing the pre-granulosa cells of the ovary. However, a substantial population (18%) of GATA4–ve cells detected in XX gonads (Fig. 1A(ii)) were also detected in XX gonads using flow cytometry could be mesonephric cells. However, IF experiments (Supp. Fig. 6) clearly identified a population of GATA4–ve cells in the developing ovary.

### 3.6. Enhancing β-catenin signalling promotes proliferation of GATA4+ve cells in XX gonads

LGR5 facilitates WNT/β-catenin signalling (Glinka et al., 2011; Koo and Clevers, 2014), promotes proliferation through the WNT signalling pathway (Koo and Clevers, 2014; Vieira et al., 2015) and is expressed in proliferating cells in the cortical region of the developing ovary (Ng et al., 2014; Rastetter et al., 2014). Moreover, loss of WNT4 results in reduced p27 and premature follicle activation, prior to transdifferentiation of pregranulosa cells to Sertoli-like cells (Maatouk et al., 2013). However, loss of p27 occurs as an indirect effect of deleting WNT4 as removal of germ cells from the...
gonad rescues mitotic arrest of granulosa cells in Wnt4 null ovaries (Maatouk et al., 2013). A previous study also demonstrated a requirement for WNT4 and RSPO1 in gonadal somatic cell proliferation, regardless of the sex of the embryo (Chassot et al., 2012). Given these findings and the propensity for β-catenin to promote proliferation and multiple observations that gain-of-function mutations in β-catenin are associated with multiple tumour types including ovarian tumours (Arend et al., 2013; Miyoshi and Henriques, 2003), we explored whether altering β-catenin signalling in the developing ovary affects proliferation of GATA4+ve somatic cells.

Initially we tested whether inhibiting WNT4 using a specific inhibitor, IWR1 (WNTi), (Chen et al., 2009) might decrease proliferation of GATA4+ve cells. Culture of E12.5 XX gonads with WNTi for 24 h significantly reduced both the percentage of p27 expressing cells (Fig. 5D ii) and the average intensity of p27 levels (Fig. 5D iii) in the GATA4+ve population. Combined, these data are consistent with a requirement for WNT/β-catenin signalling to drive somatic cell proliferation in the developing ovary.

4. Discussion

In this study, we have used in vivo and ex vivo approaches to provide novel insight into the early cellular events contributing to mouse ovary development. Our in vivo analyses demonstrate that GATA4+ve cells contribute to the supporting cells in both XX and XY gonads. In XX fetal gonads, GATA4+ve cells either express p27, enter mitotic arrest and express FOXL2, or remain proliferative and begin to express the RSPO receptor and granulosa progenitor cell marker LGR5. Moreover, although FOXL2 is dispensable for maintaining mitotic arrest, it is required to maintain p27 protein levels

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**Fig. 5. WNT/β-Catenin drives proliferation in the XX fetal gonad.** Flow cytometric analysis in E12.5 XX gonads cultured for 24 h with: A. Control (DMSO) or WNTi, and B. (i) Proliferation in GATA4+ve cells, (ii) p27+ve cells in the GATA4+ve population and (iii) p27 staining intensity in the p27+ve population. C. DMSO or GSKi (GSK3α/β inhibitor Chir99021), and D. (i) Proliferation in GATA4+ve cells, (ii) p27+ve cells in the GATA4+ve population and (iii) p27 staining intensity in the p27+ve population. Statistics are unpaired t-tests where *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 and values are mean ± SEM (n=3–4).
in these cells. In contrast, our ex vivo data demonstrate that facilitating β-catenin promotes proliferation of XX somatic cells, while blocking WNT signalling reduces XX somatic cell proliferation. These data build significantly on previously published work, to suggest a model in which pre-granulosa development is regulated at distinct levels by WNT/β-catenin and FOXL2 in the fetal ovary. We suggest that while WNT/β-catenin regulates less differentiated proliferative granulosa cell precursors, FOXL2 regulates more differentiated granulosa cells that contribute both the medullary and cortical granulosa cell populations (Fig. 6).

4.1. GATA4 expressing cells provide arrested pre-granulosa cells of the medullary follicles

Flow analyses revealed a large population of GATA4+ve cells in E11.5 XX gonads. Two thirds of these cells express p27, are arrested in G_0/G_1 of the cell cycle and gradually begin to express FOXL2 demonstrating that FOXL2 does not initiate p27 expression or mitotic arrest in the GATA4+ve/p27+ve population. However, some early FOXL2+ve cells initially appear to be negative for p27 and begin to express p27 between E11.5 and E12.5. Combined these data indicate that a positive feedback loop between FOXL2 and p27 may be involved in recruitment of the fetal granulosa cell population. Despite these observations, our analyses of Foxl2^-/- mice demonstrate that FOXL2 is not required to maintain supporting cells in mitotic arrest. In addition, since p27 is also substantially reduced in these cells, it appears that either sufficient p27 levels remain in the absence of FOXL2, or p27 is also dispensable for maintaining mitotic arrest. These observations are consistent with a lack of increased pre-granulosa cell proliferation in the fetal gonads of both Foxl2- and p27-null mice (Rajareddy et al., 2007; Schmidt et al., 2004). Given the key role of p27 in G_0/G_1 cell cycle arrest, it appears that additional factors regulate quiescence in FOXL2/p27+ve supporting cells. It is possible that the arrested supporting cells will not re-enter the cell cycle unless there is either sufficient loss of other cell cycle inhibitors such as p21CIP1 and p57KIP2, or they are exposed to a growth factor that induces their proliferation, or a combination of both.

4.2. Proliferative GATA4 expressing cells provide granulosa cell precursors in the fetal ovary

In addition to the GATA4+ve/p27+ve population, the GATA4+ve cells contribute a significant population of p27-ve cells that are highly proliferative. Significantly, these cells express LGR5 and reside primarily in the cortical region of XX gonads. Previous studies have identified LGR5+ve proliferative epithelial cells that enter mitotic arrest and upregulate FOXL2, ultimately contributing to the cortical follicles of the adult ovary (Mork et al., 2012; Ng et al., 2014; Rastetter et al., 2014). These cells are considered to contribute to pre-granulosa cells of medullary follicles until E14.5, but form a distinct population of pre-granulosa cells during E15.5 to PND4 that give rise to cortical follicles postnatally (Mork et al., 2012; Rastetter et al., 2014). However, this study also identified GATA4+ve/LGR5+ve cells that upregulate p27 at E13.5, indicating that GATA4+ve/LGR5+ve cells also contribute to the medullary granulosa cell population in the fetal ovary. Together, these data are consistent with the conclusion that GATA4 expressing cells in the XX gonad provide the granulosa precursors for both cortical and medullary follicles, but are regulated differently with respect to the choice to proliferate or enter mitotic arrest.

4.3. WNT/β-catenin signalling enhances proliferation of granulosa cell precursors

Lgr5 is aWNT target gene that potentiates β-catenin signalling in its role as an Rspo1 receptor and is expressed in proliferative epithelial cells of the ovary from E12.5 (Carmon et al., 2011; de Lau et al., 2011; Ng et al., 2014; Rastetter et al., 2014). We found that enhancing β-catenin signalling reduced p27 levels and increased proliferation in the GATA4+ve cells of the XX gonad. Consistent
with this, blocking WNT signalling increased p27 levels and reduced proliferation in the XX GATA4+ve cell population. These data indicate that the proliferation of somatic cells in the developing ovary is regulated through WNT/β-catenin signalling. By contrast FOXL2 is required for maintaining p27 in mitotically arrested XX supporting cells, indicating that it has a function that is distinct from WNT/β-catenin in the XX fetal gonad.

4.4. Modelling ovarian development through distinct WNT/β-catenin and FOXL2 functions

Combined with previous studies (Hummitzsch et al., 2013; Mork et al., 2012; Rastetter et al., 2014), our data suggest that GATA4+ve cells represent precursor supporting cells that give rise to granulosa cells of both medullary and cortical follicles in the ovary (Fig. 6). In the fetal ovary, GATA4+ve cells either, upregulate p27 and FOXL2 and directly contribute to medullary granulosa cells, or start to express LGR5, proliferate and establish a GATA4/LGR5+ve granulosa cell precursor population. Proliferation of XXY somatic cells is promoted by WNT/β-catenin, indicating that at least in part WNT/β-catenin functions to maintain proliferative granulosa cell precursors in the developing ovary. Until around E14.5 some of the GATA4/LGR5+ve cells start to express p27 and FOXL2, and maintain LGR5 in a repressed state, thereby contributing to medullary granulosa cells (Fig. 6). The remaining LGR5+ve cells give rise to cortical granulosa cells, repress LGR5 and express FOXL2 in the postnatal ovary (Fig. 6).

This model may explain the apparently differing activities of WNT/β-catenin and FOXL2 in granulosa cell development and recruitment of both the medullary and cortical follicles from the same progenitor pool. It appears likely that WNT/β-catenin and FOXL2 modulate differentiation of granulosa cells at different levels, with WNT/β-catenin maintaining a granulosa cell progenitor pool and FOXL2 functioning in the differentiation and maintenance of granulosa cells as they contribute to follicles (Fig. 6).

5. Conclusions

In this study, we provide evidence that ovarian granulosa cells of medullary and cortical follicles are derived from a common precursor cell that expresses GATA4. This is consistent with previous observations that granulosa cells are derived from GATA4 expressing cells and that GATA4 is required for granulosa cell development (Anntonen et al., 2003; Manuylov et al., 2008; Efimenko et al., 2013). In line with our findings, we propose that proliferation of GATA4 expressing cells is controlled through WNT/β-catenin signalling, while differentiation is initially marked by p27 and subsequently by FOXL2. We suggest that differentiation of granulosa cells in the developing ovary is achieved in two ways: (1) directly from precursor cell that do not establish a self-renewing stem cell population, but differentiate at the onset of ovary development via maintained expression of p27, cell cycle arrest and activation of FOXL2 expression, and (2) indirectly by initially establishing a proliferative pre-granulosa cell pool regulated by WNT4-LGR5 and β-catenin, and subsequently differentiating via unknown signals that initiate expression of p27 and FOXL2, a process that contributes both medullary and cortical follicles. The direct differentiation of granulosa cell precursors contributes to medullary follicle formation, while the indirect pathway leads to formation of both medullary and cortical follicles (Fig. 6). Further understanding of the signalling and downstream events that induce differentiation of granulosa cell precursors to promote follicular recruitment in the developing ovary will provide significant insight into events underlying establishment of the follicle reserve. This will be of importance in understanding possible aetiologies of diseases stemming from failed establishment, regulation or early demise of follicles, or the occurrence of ovarian tumours.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.02.024.

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