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Marker genes identify three somatic cell types in the fetal mouse ovary

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ABSTRACT

The two main functions of the ovary are the production of oocytes, which allows the continuation of the species, and secretion of female sex hormones, which control many aspects of female development and physiology. Normal development of the ovaries during embryogenesis is critical for their function and the health of the individual in later life. Although the adult ovary has been investigated in great detail, we are only starting to understand the cellular and molecular biology of early ovarian development. Here we show that the adult stem cell marker *Lgr5* is expressed in the cortical region of the fetal ovary and this expression is mutually exclusive to FOXL2. Strikingly, a third somatic cell population can be identified, marked by the expression of NR2F2, which is expressed in LGR5- and FOXL2 double-negative ovarian somatic cells. Together, these three marker genes label distinct ovarian somatic cell types. Using lineage tracing in mice, we show that *Lgr5*-positive cells give rise to adult cortical granulosa cells, which form the follicles of the definitive reserve. Moreover, LGR5 is required for correct timing of germ cell differentiation as evidenced by a delay of entry into meiosis in *Lgr5* loss-of-function mutants, demonstrating a key role for LGR5 in the differentiation of pre-granulosa cells, which ensure the differentiation of oogonia, the formation of the definitive follicle reserve, and long-term female fertility.

Introduction

Ovaries develop from a bipotential anlage, the genital ridges, which arise as paired structures at the ventro-medial surface of the mesonephros at around 10 days *post coitum* (dpc) in mice. Approximately half a day later, at 10.5 dpc, the male-determining gene *Sry* on the Y chromosome is up-regulated in XY genital ridges and drives differentiation into a testis. If *Sry* is not present, e.g. in an XX individual, or its expression or function is misregulated, the genital ridge will develop into an ovary (Warr and Greenfield, 2012; Wilhelm et al., 2013). The differentiation of an ovary is actively driven by a number of genes, including *Wnt4* (wingless-related MMTV integration site 4) (Vainio et al., 1999), *Rspo1* (R-spondin 1) (Parma et al., 2006) and *Foxl2* (forkhead box L2) (Ottolenghi et al., 2005; Schmidt et al., 2004).

During fetal ovarian development, primordial germ cells that have migrated to the genital ridge (Bendel-Stenzel et al., 1998) can be distinguished from ovarian somatic cells based on their morphology and through marker gene expression. Different somatic cells have been categorized into vasculature and vascular-associated cell lineages, general somatic cell lineage and pre-granulosa cell lineage based on gene expression pattern (Maatouk et al., 2012). Recent data demonstrated that two classes of pre-granulosa cells exist in the fetal mouse ovary (Mork et al., 2012; Zheng et al., 2014a). The first class, marked by the expression of FOXL2 at the early fetal stages, gives rise to granulosa cells of follicles in the ovarian medulla (Mork et al., 2012). These medullary follicles are activated before puberty and contribute to the onset of puberty and to early fertility (Zheng et al., 2014a). The second class of pre-granulosa cells, for which no marker gene has yet been identified, resides in the cortical region of the fetal ovary (Mork et al., 2012). These cells will differentiate into granulosa cells of the cortical follicles that are gradually activated and therefore constitute the definitive pool of primordial follicles for the entire reproductive lifespan of the organism (Monget et al., 2012). All granulosa cells in the postnatal ovary, i.e. granulosa cells of the medullary and cortical

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follicles, are marked by the expression of FOXL2 (Pisarska et al., 2004). In addition to granulosa cells, the developing ovary contains steroidogenic theca cells, the origin of which is even less clear. They are first observed after birth when a follicle consists of two or more layers of granulosa cells and it is assumed that signals from the growing follicles stimulate the differentiation of theca cells from unidentified progenitor populations (Young and McNeilly, 2010). Similar to the cortical pregranulosa cells, markers specific for theca progenitor cell populations have not yet been identified.

Lgr5 encodes a G-protein-coupled receptor that is related to the hormone receptors for thyroid-stimulating hormone, follicle-stimulating hormone and luteinizing hormone. They belong to the large, G-protein-coupled, 7-transmembrane family of proteins (Hermey et al., 1999). *Lgr5* was identified as a WNT target gene in colon carcinoma cell lines and patient-derived colorectal cancer samples (Van der Flier et al., 2007) and shown to mark adult stem cells in the small intestine, colon, stomach, and hair follicles (Barker et al., 2010, 2007; Jaks et al., 2008) and, more recently, as a marker of stem cells in the ovarian surface epithelium, which contains a cancer-prone stem cell niche (Flesken-Nikitin et al., 2013; Ng et al., 2014). For a long time, LGR5 was considered to be an orphan receptor, with ligands remaining unknown. However, recently it has been shown that LGR5 binds R-spondins with high affinity and enhances RSPO/WNT/β-catenin signaling (Carmon et al., 2011; Chen et al., 2013; de Lau et al., 2011; Ruffner et al., 2012). While the function of LGR5 in adult stem cells has been investigated in great detail, its role during embryogenesis is less clear. Deletion of *Lgr5* in vivo results in neonatal lethality associated with a malformation of the tongue among other pathologies (Kinzel et al., 2014; Morita et al., 2004). In addition, the importance of both LGR5 and the related factor LGR4 in embryonic gut, kidney and skin development has been reported recently (Kinzel et al., 2014). In contrast, the involvement of these factors in gonad development is unknown.

Here we show that WNT4 and RSPO1 signaling up-regulates *Lgr5* expression in the developing ovary as part of an apparent feed-forward WNT signaling regulatory network. LGR5 in turn is necessary for proper germ cell differentiation, consistent with its role as a RSPO1 receptor. Furthermore, the expression of LGR5, FOXL2 and another transcription factor, NR2F2, in ovarian somatic cells is mutually exclusive, demonstrating that at least three different somatic cell precursors exist in the fetal ovary. Moreover, using lineage tracing, we demonstrate that LGR5-positive cells give rise to cortical granulosa cells, which are essential for the formation of the follicles, thereby establishing the ovarian reserve.

Materials and methods

Mouse strains

Wild-type C57BL/6 mice were obtained from Monash University Central animal services. *Lgr5* knock-in mice (*Lgr5*$^{tm1(cre/ERT2)Cle}$/J (*Lgr5-eGFP-IRES-CreERT2*), (Barker et al., 2007)) and Rosa26-lacZ reporter mice ((*Gt*(*ROSA*)*26Sor*^{tm1Sor}/J, (Soriano, 1999)) were obtained from the Jackson laboratory. *Wnt4*$^{-/-}$ and *Rspo1*$^{-/-}$ mice have been described before (Chassot et al., 2008; Vainio et al., 1999) and were maintained on a mixed 129/C57BL6 genetic background. Mouse embryos were collected from timed matings with noon of the day on which the mating plug was observed designated 0.5 days *post coitum* (dpc). For more accurate staging, the tail somite (ts) stage of the embryo was determined by counting the number of somites posterior to the hind limb (Hacker et al., 1995). Using this method, 10.5 dpc corresponds to approximately 8 ts, 11.5 dpc to 18 ts, and 12.5 dpc to 30 ts. The genetic sex of the embryos was determined by PCR for pseudoautosomal genes on the X and the Y chromosome (McFarlane et al., 2013). Protocols and use of animals conformed to the National Health and Medical Research Council/Commonwealth Scientific and Industrial Research Organization/Australian Agricultural Council Code of Practice for the Care and Use of Animals for Experimental Purposes and were approved by the Monash Animal Research Platform Committee on Ethics in Animal Experimentation.

Quantitative real-time RT-PCR (qRT-PCR)

qRT-PCR using SYBR green (Invitrogen) was performed as described previously (Svingen et al., 2009; van den Bergen et al., 2009). qRT-PCR at all stages was performed on gonad-only samples with mesonephroi removed. Briefly, 200 ng of input RNA, pooled from like samples, was subject to cDNA synthesis with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) as per manufacturer's instructions. 1 μl of the resultant cDNA reaction was used in a 20 μl qRT-PCR mastermix containing 1 × SYBR Green PCR Master Mix (Applied Biosystems) and 175 nM each of the forward and reverse primers. 5 μl triplicate reactions were run in 384 well plates on a Viia7 Real Time PCR System (Applied Biosystems) as technical replicates. Products were analyzed by gel electrophoresis then cloned and sequenced to verify specificity of amplified sequence. Primer efficiencies were determined, which were similar for all primer pairs used in this analysis. Gene expression was normalized to *Sdha* (Svingen et al., 2009) and qRT-PCR analysis was performed on at least three independent biological samples. As positive controls, *Foxl2* as an ovarian-enriched gene, and *Amh* as a testicular gene were included (data not shown) as described before (Chen et al., 2012). For each gene, data sets were analyzed for statistically significant differences between XX and XY expression levels using a two-tailed, unpaired *t*-test with confidence intervals set at 95%. Primers used are described in Table S1.

Immunofluorescence

Embryos were retrieved at different stages during embryonic development and either processed whole or gonads dissected and fixed in 4% paraformaldehyde (PFA) in PBS at 4 °C. Embryos and isolated gonads were embedded in paraffin and immunofluorescence performed as described previously (Wilhelm et al., 2005). Primary antibodies used for this study were goat anti-EGFP (Abcam) used at 1:200, chicken anti-EGFP (Abcam) used at 1:200, mouse anti-EGFP (Santa Cruz) used at 1:200, goat anti-MVH (R&D Systems) used at 1:400, rabbit anti-FOXL2 (Wilhelm et al., 2009) used at 1:400, rabbit anti-SOX9 (Wilhelm et al., 2005) used at 1:200, mouse anti-NR2F2 (Persus Proteomics) used at 1:300, goat anti-β-galactosidase (BioGenesis) used at 1:200, mouse anti-OCT4 (Santa Cruz) used at 1:50, rabbit anti-SYCP3 (Abcam) used at 1:200, rabbit anti-CD31 (PECAM1; Abcam) used at 1:50 and mouse anti-E-cadherin (BD Transduction Laboratories) used at 1:300. Secondary antibodies used were donkey anti-goat Alexa 488, goat anti-chicken Alexa 488, donkey anti-mouse Alexa 488, goat anti-mouse Alexa 546, donkey anti-rabbit Alexa 488, donkey anti-rabbit Alexa 594 and goat anti-rabbit Alexa 647 all obtained from Invitrogen and used at 1:300, as well as 4′,6-diamidino-2-phenylindole (DAPI, from Molecular Probes) used at 1:2000 to visualize nuclear DNA in immunofluorescence using a Zeiss LSM 510 Meta confocal microscope at the Australian Cancer Research Foundation Dynamic Imaging Centre for Cancer Biology, University of Queensland and a Nikon C1 confocal microscope running with NIS Elements Software (Nikon, Tokyo, Japan) at the Monash Micro Imaging Facility.

Section in situ hybridization (ISH)

In situ probes for *Lgr4* (NM_172671), *Lgr5* (NM_010195), and *Lgr6* (NM_001033409) were cloned by RT-PCR from RNA prepared from whole mouse embryos at 13.5 dpc. Primers used to generate gene-specific ISH probes are listed in Table S1. Section ISH was carried out as described previously (Wilhelm et al., 2007) and imaged using an Olympus BX-51 microscope.

Lineage tracing

To lineage-trace *Lgr5*-expressing cells throughout development, we made use of the Cre-ERT2 gene "knocked-in" to the *Lgr5* locus and Rosa26-lacZ reporter mice (*Gt(ROSA)26Sor*^{tm1Sor}/J, (Soriano, 1999)). *Lgr5*$^{-}$/$^{+}$ mice were bred with Rosa26-lacZ mice and pregnant females were injected with 2 mg tamoxifen (20 mg/ml) per 40 g body weight at 13.5, 15.5 and 17.5 dpc. To counteract the mixed estrogen agonist effects of tamoxifen injections, which can result in late fetal abortions in pregnant mice, progesterone was co-injected at 13.5 dpc. The ovaries from offspring were collected at 2 and 4 weeks postnatally and either stained for β-galactosidase activity as described previously (Wilhelm and Englert, 2002), embedded in paraffin, sectioned and counterstained with eosin, or embedded in O.C.T. compound (VWR International), cryosectioned and used in immunofluorescence to detect β-galactosidase protein.

Results

Lgr5 expression is enriched in the ovary and dependent on WNT signaling

Lgr5 was previously identified as a gene with high expression in the developing mouse ovary (Jameson et al., 2012; Nef et al., 2005). To analyze its expression during mouse sex determination and gonad differentiation in greater detail we performed quantitative real-time RT-PCR (qRT-PCR) on mouse fetal gonads from 11.5 dpc, the time of sex determination, to 17.5 dpc, shortly before birth. *Lgr5* was expressed at very low levels at 11.5 dpc in both XX and XY genital ridges (Fig. 1A). By 12.5 dpc, *Lgr5* expression was elevated in both XX and XY samples but significantly more so in the developing ovary compared to testis. Subsequently, *Lgr5* expression was down-regulated in the developing testis, but further up-regulated and maintained at high levels in the ovary until at least 17.5 dpc (Fig. 1A).

Due to the lack of a suitable antibody to detect mouse LGR5 protein, we made use of an *Lgr5* EGFP knock-in mouse line (*Lgr5*$^{tm1(cre/ERT2)Cle}$/J) that expresses EGFP in place of *Lgr5*. In the phenotypically normal *Lgr5*$^{+/-}$ mice, EGFP recapitulates *Lgr5* expression and can be monitored by immunofluorescence (IF) (Barker et al., 2007). EGFP was not detected in the developing testis at any of the stages investigated in *Lgr5*$^{-/+}$ mice (Fig. S1). By contrast, consistent with the increase in *Lgr5* transcription that was detected using qRT-PCR, EGFP-positive cells were detected by IF from 12.5 dpc onwards in the developing ovaries of *Lgr5*$^{-/+}$ mice, with expression becoming increasingly restricted to the cortical region of the ovary (Fig. 1B). This analysis was also supported using section in situ hybridization (ISH) of 11.5 dpc to 13.5 dpc XX and XY mouse embryos (Fig. S2), which confirmed that EGFP expression recapitulates endogenous *Lgr5* expression during gonad development. The expression pattern observed in the section ISH suggested that *Lgr5* was restricted to somatic cells in the ovary (Fig. S2). Confirming this, co-IF of EGFP and mouse vasa homologue (MVH), a marker for germ cells, revealed that MVH and LGR5 mark distinct cell populations in 14.5 dpc and 18.5 dpc ovaries, demonstrating that LGR5 is restricted to somatic cells in the developing ovaries (Fig. 1C).

Lgr5 has been identified as a WNT target gene in colon carcinoma cell lines (Van der Flier et al., 2007). To test if *Lgr5* expression is also dependent on WNT signaling during gonadal development, we compared its expression by qRT-PCR in *Wnt4*$^{-/-}$ (Fig. 2A), *Rspo1*$^{-/-}$ (Fig. 2B), and control ovaries at 12.5 dpc. In both *Wnt4* and *Rspo1* knockout ovaries, *Lgr5* expression was statistically significantly reduced when compared to control ovaries, demonstrating that WNT signaling is also necessary for up-regulating *Lgr5* expression in the developing ovary.

Lgr5 is necessary for the differentiation of XX germ cells

We have shown that *Lgr5* is expressed in the cortical region of the ovary (Fig. 1B), where germ cells survive and form primordial follicles after birth. This suggested a role for LGR5 in contributing to the niche for germ cell maintenance and maturation during ovarian development. In addition, LGR5 protein is a RSPO1 receptor that is necessary to enhance WNT/RSPO signaling (Carmon et al., 2011; Chen et al., 2013; de Lau et al., 2011; Ruffner et al., 2012). In both *Wnt4* and *Rspo1* mutant ovaries, germ cell differentiation and survival is affected (Chassot et al., 2011, 2008; Tomizuka et al., 2008; Vainio et al., 1999). We therefore analyzed germ cell differentiation and maintenance in *Lgr5*$^{-/-}$ ovaries by IF. Germ cell differentiation in the embryonic ovary is characterized by entry into meiosis in an anterior-to-posterior wave starting at around 13.5 dpc (Bullejos and Koopman, 2004; Menke et al., 2003). IF at 14.5 dpc demonstrated that most, if not all, germ cells had entered meiosis in the wild-type ovary as indicated by their high expression of SYCP3 and undetectable levels of the pluripotency marker OCT4 throughout the ovary (Fig. 3A, top left panel). In contrast, in the *Lgr5*$^{-/-}$ ovary at 14.5 dpc only approximately half of the primordial germ cells had entered meiosis (Fig. 3A, bottom left panel), demonstrating that entry into meiosis is delayed in the absence of *Lgr5*. By 16.5 dpc though, we did not detect any nuclear OCT4 in the *Lgr5*$^{-/-}$ ovary, and most germ cells expressed phospho-γH2.AX, showing that the germ cells have entered meiosis (Fig. 3A, right panels). Although meiotic entry of germ cells was delayed, the differentiation of FOXL2-positive cells and EGFP-expressing *Lgr5*$^{-/-}$ somatic cells (Fig. S3) appeared normal. In addition, we did not detect any other defects in *Lgr5*$^{-/-}$ fetal ovaries.

To examine germ cell numbers in more detail, we carried out IF with the germ cell marker MVH (Toyooka et al., 2000) on 14.5 and 18.5 dpc wild-type and *Lgr5*$^{-/-}$ ovaries. While in some samples the number of germ cells in Lgr5$^{-/-}$ ovaries appeared less than in control ovaries (Fig. 3B), this phenotype was variable with some samples containing normal germ cell numbers (Fig. S4A). These results contrast to the consistent germ cell loss observed in *Wnt4*$^{-/-}$ (Vainio et al., 1999) or *Rspo1*$^{-/-}$ (Chassot et al., 2011, 2008; Tomizuka et al., 2008) ovaries. In addition, qRT-PCR for *Axin2*, a known target of WNT/RSPO signaling (Jho et al., 2002), demonstrated that, in contrast to the *Wnt4* (Chassot et al., 2008) and *Rspo1* (Chassot et al., 2011, 2008) knockout, *Axin2* expression is unchanged in the absence of *Lgr5* (Fig. S4B). Since *Lgr5*$^{-/-}$ mice die neonatally (Morita et al., 2004), analysis of postnatal germ cells was not possible.

Lgr4 but not Lgr6 is expressed in developing mouse gonads

The variation we observed in germ cell numbers in *Lgr5*$^{-/-}$ ovaries could be due to compensatory mechanisms involving closely related genes. Indeed, *Lgr5* is a member of one of the largest families of seven-transmembrane domain receptors, with *Lgr4* and *Lgr6* being the closest related family members (Hsu et al., 2000). To test if *Lgr4* and/or *Lgr6* are expressed in the developing gonads, we performed qRT-PCR (Fig. 4A and B) and section ISH (Fig. 4C). qRT-PCR on 11.5 to 15.5 dpc XX and XY wild-type gonads demonstrated that *Lgr4* is

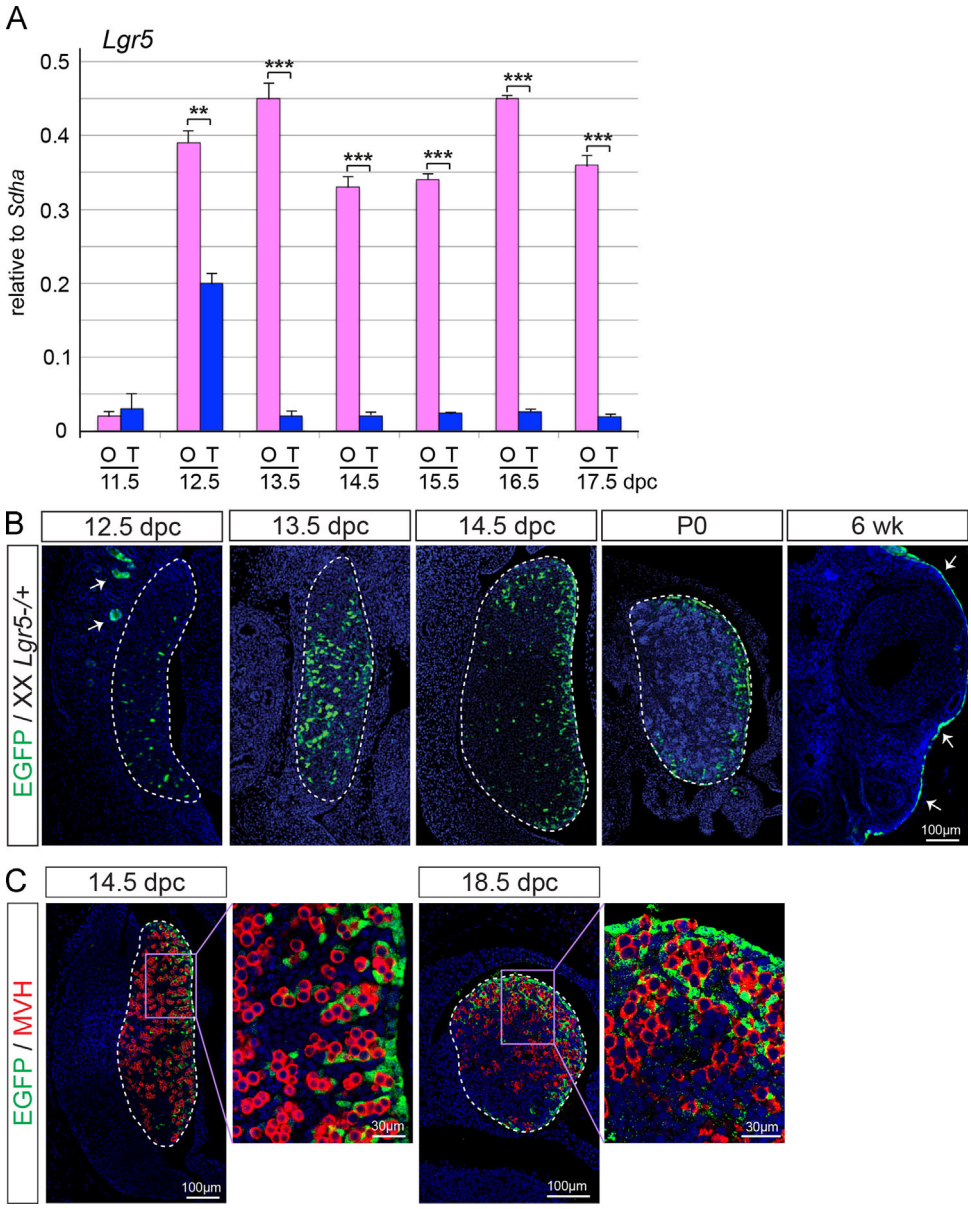

Fig. 1. *Lgr5* expression is ovarian-enriched during mouse gonad development. (A) qRT-PCR of mouse embryonic ovaries (O) and testes (T) from 11.5 to 17.5 dpc demonstrates ovarian-enriched expression of *Lgr5*. At least three independent biological replicates were performed for qRT-PCR analysis with individual experiments performed in triplicate on RNA obtained from pooled gonads from 3 to 4 littermates and expression levels shown relative to *Sdha* (mean + SEM of at least three independent experiments; two-tailed, unpaired *t*-test; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$). (B) IF for EGFP (green) on sagittal section of 12.5 dpc to 6 weeks postnatally (6wk) XX $Lgr5^{-/+}$ embryos. EGFP is knocked-in to the *Lgr5* locus and mimics endogenous *Lgr5* expression. Ovaries are demarcated with dotted line; arrows in the left panel mark EGFP expression in mesonephric tubules, and arrows in the right panel indicate EGFP expression in ovarian epithelial cells. P0: day of birth. Scale bar, 100 µm. (C) Co-IF for EGFP (green) and the definitive germ cell marker MVH (red) on sections of 14.5 and 18.5 dpc XX $Lgr5^{-/+}$ embryos demonstrating that EGFP expression in somatic cells. Ovaries are demarcated with dotted line; the second and forth panel show a higher magnification of the area marked by a square in the first and third panel, respectively. Scale bars, 100 µm (left and right panels) and 30 µm (middle panel).

expressed at high level in 11.5 dpc testes and ovaries. At 12.5 dpc expression levels were reduced, and from 13.5 dpc *Lgr4* was expressed expression at slightly higher levels in the ovary compared to the testis (Fig. 4A). *Lgr6* showed a similar pattern of increasingly ovarian-enriched expression over the course of gonad development albeit at overall lower levels as compared to *Lgr4*.

To confirm this expression and to identify the cell types that express *Lgr4* and *Lgr6* we carried out section ISH on 13.5 dpc whole mouse embryos. *Lgr4* expression was detected in somatic but not germ cells of the ovary and the testis (Fig. 4C, left and middle panel), supporting the hypothesis that it acts redundantly with *Lgr5*. In contrast, *Lgr6* expression was undetectable by ISH in 13.5 dpc ovaries and testes (Fig. 4C, right panel), confirming the low overall expression levels detected by qRT-PCR. This was not due to inefficiency in achieving a robust ISH signal, as robust expression of *Lgr6* was detected in the hair follicles of the whiskers (Fig. 4C, right panel, red arrows) as has been described before (Snippert et al., 2010).

The fetal ovary contains at least three different somatic cell precursors

Others and we have shown that there is molecular regionalization of the embryonic mouse ovary into distinct cortex and medulla (Chen et al., 2012; Yao et al., 2004). We detected *Lgr5* expression in the cortex (Fig. 1B), whereas FOXL2 expression has been described in the medullary region (Chen et al., 2012). To directly compare the expression of these two ovarian somatic cell genes, we

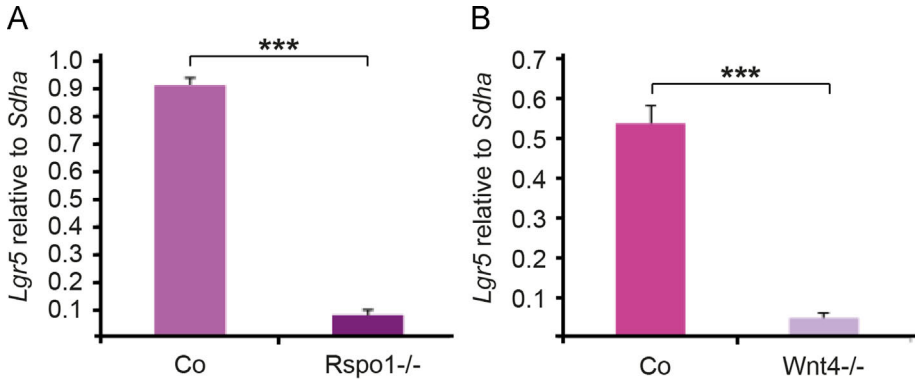

Fig. 2. *Lgr5* is not expressed in *Rspo1*- and *Wnt4*-null ovaries. qRT-PCR of control ovaries compared to *Rspo1*$^{-/-}$ (A) and *Wnt4*$^{-/-}$ (B) ovaries at 12.5 dpc demonstrates a lack of *Lgr5* expression in mutant ovaries. At least three independent experiments were performed with individual experiments performed in biological triplicate on RNA obtained from pooled gonads from 3 to 4 littermates and expression levels shown relative to *Sdha* (mean +SEM of at least three independent experiments; two-tailed, unpaired *t*-test; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

performed co-IF for EGFP and FOXL2 in 12.5 dpc to 18.5 dpc *Lgr5*$^{-/+}$ ovaries. Interestingly, EGFP and FOXL2 expression were almost completely mutually exclusive at the cellular level at all time points investigated (Fig. 5A and data not shown), suggesting that they mark different somatic cell types. We detected a few cells that expressed both EGFP and FOXL2 especially at the early stages, but in all cases the expression of one of the two proteins appeared very low (arrows in Fig. 5A, third panel).

We next compared the expression of EGFP and FOXL2 with the expression of NR2F2 (also known as nuclear chicken ovalbumin upstream promoter-transcription factor, COUP-TFII), which has been shown to be expressed in theca cells of human ovaries (Sato et al., 2003). Surprisingly, double IF for NR2F2 and FOXL2 as well as NR2F2 and EGFP in 13.5 dpc and 15.5 dpc wild-type ovaries showed that the expression of these factors is mutually exclusive at a cellular level (Fig. 5B and C, and data not shown). LGR5, FOXL2 and NR2F2 therefore represent markers that distinguish between three different somatic cells in the mouse ovary. Germ cells can be identified by the DNA stain DAPI, based on their characteristic large, round nuclei. When DAPI was combined with triple IF for EGFP, FOXL2 and NR2F2 most remaining cells that did not express EGFP, FOXL2 or NR2F2 had characteristic large round nuclei, indicating that they are germ cells (Fig. 5C, right panel).

The expression pattern of NR2F2 resembled that described for the transcription factor MAFB, which is expressed in cells closely associated with vascular cells (Maatouk et al., 2012). We therefore performed double-IF for NR2F2 and PECAM1, a marker for endothelial cells and germ cells. This analysis showed that NR2F2 is, similar to MAFB, expressed in cells in close proximity to vascular endothelial cells (Fig. 6A), suggesting that the same cells express these two markers. To examine in more detail whether there are any ovarian somatic cells that do not express FOXL2, EGFP (*Lgr5*) and NR2F2, we combined the triple IF for EGFP (green), FOXL2 (light blue), and NR2F2 (purple), marking ovarian somatic cells, with IF for MVH (red), labeling germ cells, on 14.5 and 16.5 dpc *Lgr5*$^{-/+}$ ovaries (Fig. 6B). Interestingly, at 14.5 dpc there were still cells throughout the ovary, and especially at the poles, that did not express any of the four factors (Fig. 6B, arrow heads). Based on the shape of the nuclei these cells appeared to be somatic cells and not germ cells. However, by 16.5 dpc, all ovarian cells expressed either EGFP, FOXL2, NR2F2 or MVH (Fig. 6B), except a few ovarian surface epithelial cells at the base of the ovary (Fig. 6B, arrow heads).

Lgr5-positive cells give rise to cortical adult granulosa cells

Mork et al. (2012) recently showed that FOXL2-positive cells in the fetal ovary give rise to the granulosa cells of the medullary follicles in the postnatal ovary. They also suggested that somatic cells of the ovarian surface epithelium are precursor cells of the cortical follicles, similar to what has been shown in other species (Hummitzsch et al., 2013; Sawyer et al., 2002). Based on the expression pattern we described for LGR5 and FOXL2, we hypothesized that these precursor cells are LGR5-positive. To test this hypothesis we made use of the inducible *CreERT* knocked-in to the *Lgr5* locus of the *Lgr5-eGFP-IRES-CreERT2* mice. We crossed these mice with *Rosa26-lacZ* mice, injected tamoxifen to activate *Cre* recombinase at 17.5 dpc, and analyzed β-galactosidase activity in the ovaries of offspring at 4 weeks of age. While no β-galactosidase activity was detected in *Lgr5*$^{+/+}$:R26LacZ (Fig. 7A, left panel), strong β-galactosidase activity was observed in a subset of granulosa cells of cortical follicles (Fig. 7A, middle and right panel), and in some cells of the ovarian surface epithelium (Fig. 7A, arrow in middle panel), indicating that LGR5-positive cells at 17.5 dpc gave rise to granulosa cells of the cortical follicles.

Next, we asked whether *Lgr5*-positive cells at earlier stages also give rise to cortical granulosa cells in the postnatal ovary. To answer this question, *Lgr5-eGFP-IRES-CreERT2* mice crossed with *R26-LacZ* mice were injected with tamoxifen at 13.5 dpc and β-galactosidase detected by IF on 4-week-old ovaries from offspring. No β-galactosidase signal was detected in control ovaries (Fig. 7B, left panel), whereas β-galactosidase was present in cortical granulosa cells of *Lgr5*$^{-/+}$:R26lacZ ovaries (Fig. 7B, right panel), indicating that *Lgr5*-positive cells as early as 13.5 dpc represent cortical granulosa precursor cells (Fig. 7B).

Discussion

We have identified three factors, LGR5, FOXL2 and NR2F2, that mark specific ovarian somatic cell precursors, of which *Lgr5*-positive somatic cells differentiate into cortical granulosa cells of the adult ovary. In addition, we show that loss of *Lgr5*, a well-known adult stem cell marker (Barker et al., 2010, 2007; Jaks et al., 2008), results in a delay of germ cell entry into meiosis and is therefore involved in fetal ovarian development, most likely in its role as RSPO1 receptor.

Three distinct somatic precursor cell populations exist in the fetal ovary

The study of mouse fetal ovarian development has been hampered by the fact that even after sex is determined and the female program of gene expression in XX genital ridges is activated, no major morphological changes occur in the fetal ovary

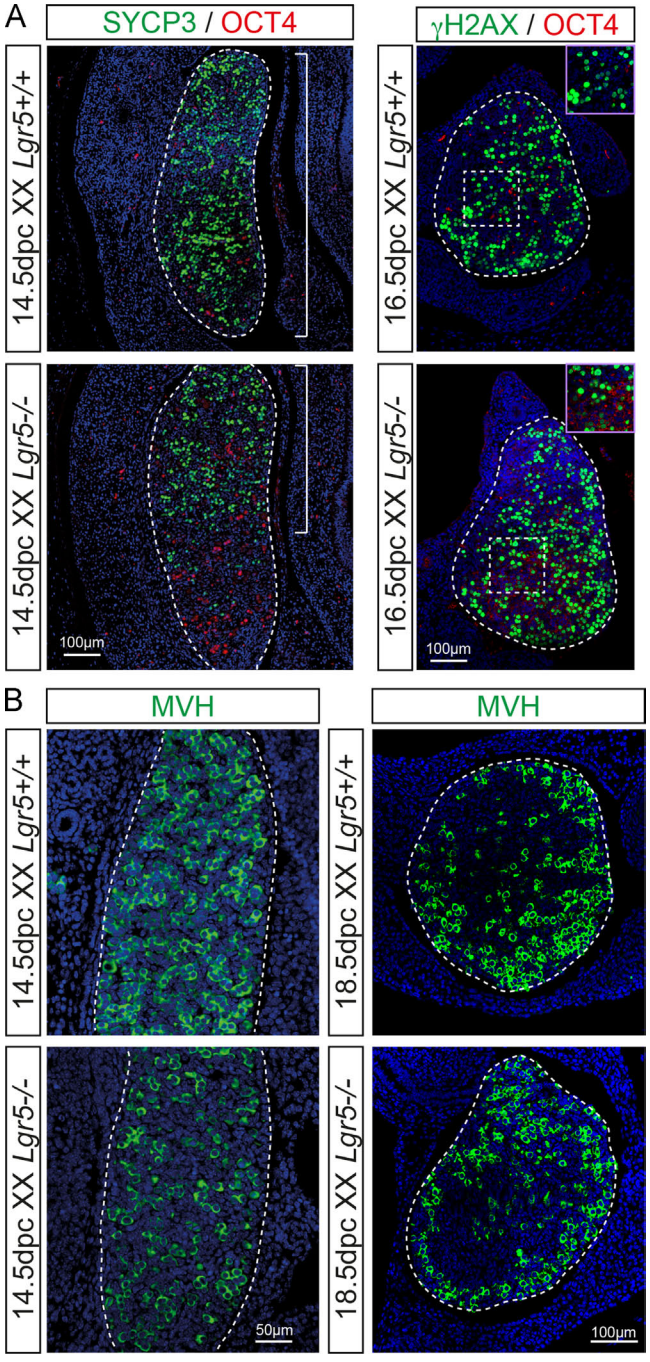

Fig. 3. Loss of *Lgr5* results in a delay of germ cell entry into meiosis. (A) Double IF for left panel: SYCP3 (green; meiotic germ cells) and OCT4 (red; pluripotency marker of germ cells), or right panel: phospho-γH2AX (green) and OCT4 (red) on sagittal sections of gonads from control and *Lgr5*-null fetuses at 14.5 dpc (left panel) and 16.5 dpc (right panel). While almost all germ cells in the control ovaries (left upper panel) have up-regulated expression of SYCP3 and repressed OCT4, entry into meiosis is delayed in *Lgr5*-null ovaries as indicated by fewer SYCP3 positive cells and more OCT4 positive cells (bottom left panel). By 16.5 dpc no nuclear OCT4 expression was detected and most germ cells have entered meiosis in wild-type as well as mutant ovaries. Ovaries are demarcated with dotted lines; the extent of the anterior-to-posterior wave of cells entering meiosis is indicated by the brackets. The insets in the right panels show the area marked by a square at higher magnification. Scale bar, 100 µm. (B) IF for the germ cell marker MVH (green) on 14.5 and 18.5 dpc XX control and *Lgr5*$^{-/-}$ fetuses suggests a slight reduction in germ cells in mutant ovaries. Ovaries are demarcated with dotted lines. Scale bar, 50 µm (left panels) and 100 µm (right panels).

for a few days. In contrast, Sertoli cells differentiate in the developing testis and rapidly assemble around clusters of germ cells and undergo mesenchymal-to-epithelial transition to form

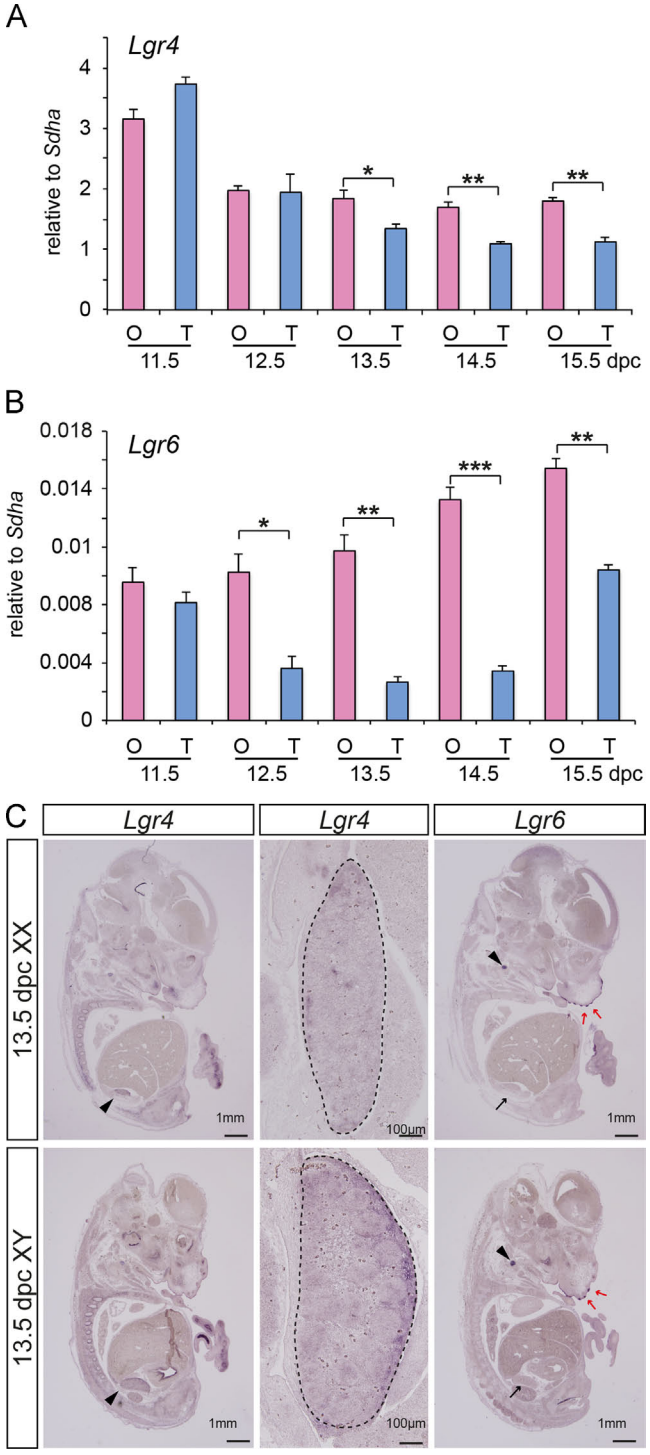

Fig. 4. *Lgr4*, but not *Lgr6*, is expressed in mouse embryonic gonads. qRT-PCR for *Lgr4* (A) and *Lgr6* (B) with RNA isolated from 11.5 to 15.5 dpc mouse embryonic ovaries (O) and testes (T) demonstrates the expression of *Lgr4* in both ovaries and testes at all stages, with higher expression in the ovary from 13.5 dpc onwards. In contrast, *Lgr6* is expressed at very low levels with ovarian-enriched expression from 12.5 dpc. At least three independent biological replicates were performed for qRT-PCR analysis with individual experiments performed in triplicate on RNA obtained from pooled gonads from 3 to 4 littermates and expression levels shown relative to *Sdha* (mean + SEM of at least three independent experiments; two-tailed, unpaired *t*-test; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$). (C) ISH on sagittal sections of 13.5 dpc mouse embryos confirmed the expression of *Lgr4* in somatic cells of ovary and testis (arrowheads in left panels and middle panels), whereas no *Lgr6* expression was detected in the developing gonads (arrows in right panels). However, *Lgr6* expression was detected in the hair follicles of the whiskers (red arrows in right panels) and the thymic primordium (arrow head in right panels). Scale bars, 1 mm (left and right panels) and 100 µm (middle panels).

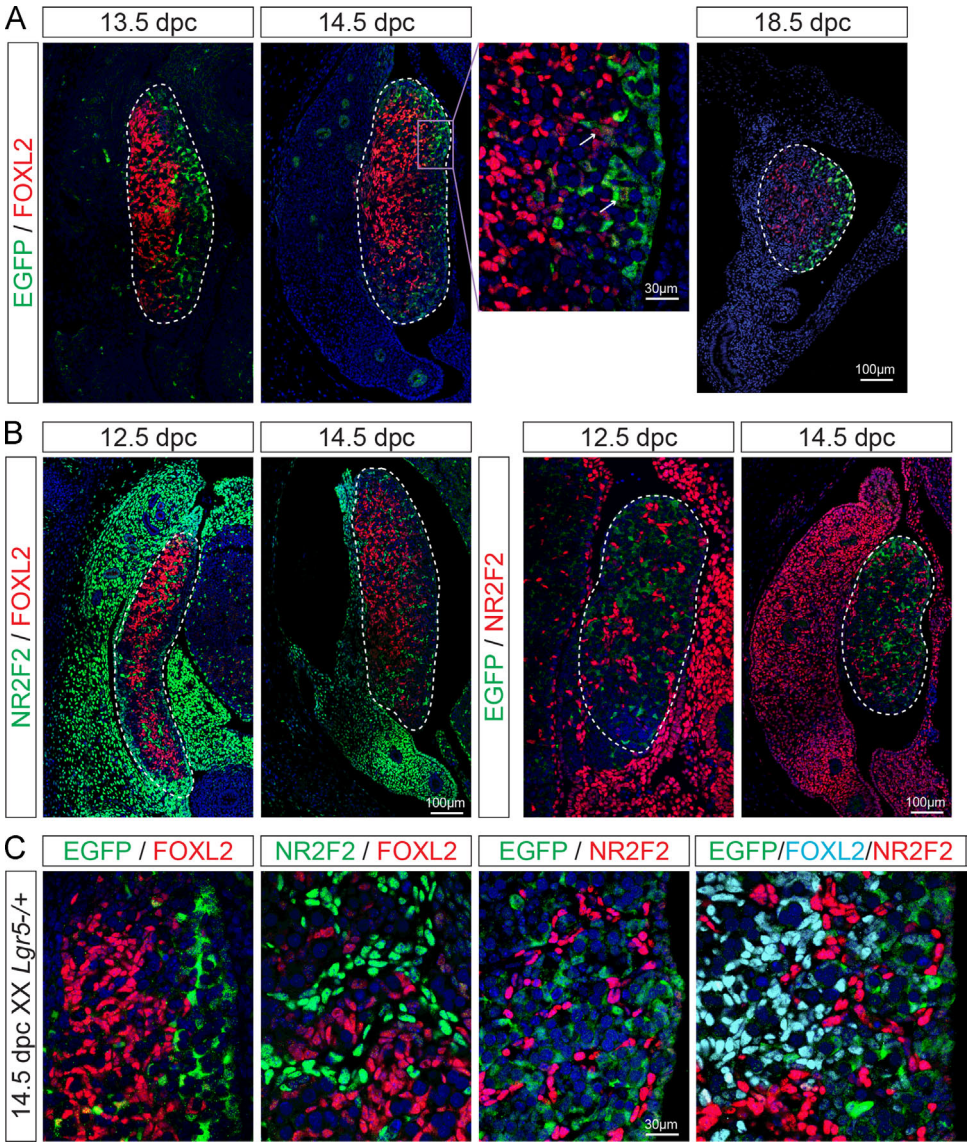

Fig. 5. EGFP, FOXL2 and NR2F2 mark different ovarian somatic cells. (A) Co-IF for FOXL2 (red) and EGFP (green) of 13.5 to 18.5 dpc XX *Lgr5*$^{-/+}$ mouse fetuses demonstrated that the expression of these two genes is mutually exclusively in the developing ovary. In the few cells (arrows in third panel) that show co-expression one of the factors is always expressed at very low levels. Ovaries are demarcated with dotted lines. The third panel shows higher magnification of the area marked with a rectangle in the second panel. Scale bars, 100 µm (first, second and last panel) and 30 µm (third panel). (B) Co-IF of NR2F2 (green, two left panels) with FOXL2 (red), or NR2F2 (red, two right panels) with EGFP (green), of 12.5 and 14.5 dpc XX *Lgr5*$^{-/+}$ mouse fetuses shows no overlap of expression. Ovaries are demarcated with dotted lines. Scale bars, 100 µm. (C) High magnifications of double-IF of 14.5 dpc XX *Lgr5*$^{-/+}$ mouse fetuses: first panel: EGFP (green) with FOXL2 (red); second panel: NR2F2 (green) with FOXL2 (red); third panel: EGFP (green) with NR2F2 (red). The last panel on the right shows triple-IF of all three factors (EGFP, green FOXL2, light blue; NR2F2, red), clearly demonstrating mutually exclusive expression. Scale bar, 30 µm.

testis cords (Kanai et al., 1991, 1992). Shortly after, steroidogenic fetal Leydig cells differentiate within the interstitium between testis cords (Svingen and Koopman, 2013). These morphological changes not only made it possible to distinguish between different testicular somatic cell precursors based on their cell morphology and position within the testis, but also to identify factors that could be used as exclusive markers to follow the differentiation and maturation of each cell type. Different somatic cell types in the fetal ovary have been classified into pre-granulosa cells marked by FOXL2, vasculature-associated somatic cells marked by MAFB and general somatic cells (Maatouk et al., 2012). In this study we extend these findings through the identification of *Lgr5*, which is specifically expressed by the second class of pre-granulosa cells, and NR2F2, as a possible second marker for vasculature-associated somatic cells.

It is generally thought that the genital ridge contains at least three bipotential precursor cell lineages (Svingen and Koopman, 2013): (i) Precursors of the supporting cells that start to express *Sry* and differentiate into Sertoli cells in the XY genital ridge, or express *Foxl2* and differentiate into granulosa cells in the XX genital ridge; (ii) Steroidogenic precursor cells which differentiate into Leydig cells in a testis or theca cells in the ovary; and (iii) primordial germ cells that give rise to spermatogonia and oogonia in testes and ovaries, respectively. Our data combined with recent studies (Mork et al., 2012; Zheng et al., 2014a) now demonstrate that there are at least four precursor cell types in the fetal ovary, including two different populations of supporting cell precursors, *Foxl2* and *Lgr5*-positive cells, steroidogenic cell precursor and primordial germ cells. It is not known if a second population of supporting cell precursors exist in the testis and whether they

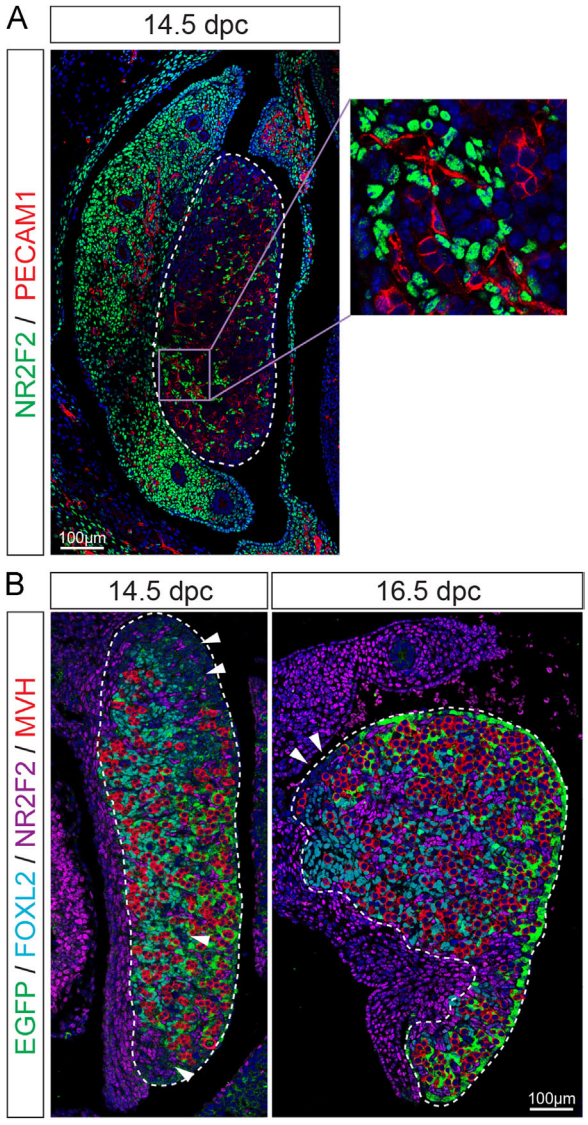

Fig. 6. Triple-negative somatic cells disappear by 16.5 dpc. (A) Co-IF for NR2F2 (green) and PECAM1 (red) of 14.5 dpc XX wild-type ovary showed that NR2F2 is expressed in cells in close proximity to vascular endothelial cells. Ovaries are demarcated with dotted lines. The right panel shows higher magnification of the area marked with a rectangle in the left panel. Scale bar, 100 µm. (B) Co-IF for EGFP (green), FOXL2 (light blue), NR2F2 (pink) and MVH (red) of 14.5 dpc (left panel) and 16.5 dpc (right panel) XX *Lgr5*$^{-/+}$ fetuses showed that at 14.5 dpc a number of ovarian somatic cells (arrow heads), while at 16.5 dpc only a few ovarian surface epithelium cells are triple negative. Ovaries are demarcated with dotted lines. Scale bars, 100 µm.

might contribute a distinct Sertoli cell population. One possibility comes from our work demonstrating that production of prostaglandin D_2 by the founding population of Sertoli cells is able to recruit other somatic cells to express SOX9 (Wilhelm et al., 2005), potentially providing a second source of Sertoli cell precursors. However, the location of these cells remains unknown and the number of cells recruited by this pathway is very small, so it is unlikely that these cells recruited by prostaglandin D_2 represent the second supporting cell lineage, equivalent to the ones marked by *Lgr5* in the developing ovary.

In addition to the *Lgr5* population, we identified a population of ovarian cells marked by the expression of NR2F2, and possibly MAFB. These cells could be either mesenchymal cells or may differentiate into theca cells. At present this is not possible to demonstrate as *Nr2f2*-null mice die before 10.5 dpc, preventing the analysis of ovarian development in these mice (Pereira et al., 1999). However, it is of significant interest that *Nr2f2*-heterozygous ovaries have a reduced ability to produce sex steroids (Takamoto et al., 2005), supporting the hypothesis that this transcription factor is involved in theca cell function. In addition, NR2F2 has been shown to play a role in Leydig cell differentiation, the steroidogenic lineage in the testis, and regulate Leydig cell steroidogenesis (Defalco et al., 2011; Qin et al., 2008; van den Driesche et al., 2012), further corroborating the concept that in females NR2F2 is involved in the differentiation of the steroidogenic theca cells.

Lgr5 marks cortical granulosa cell precursors

There are two types of ovarian follicles in mammals (Zheng et al., 2014b). First, medullary follicles are collectively activated before puberty and contribute to puberty and early fertility up to about 5 weeks postnatally (Zheng et al., 2014a). Second, cortical follicles are activated throughout life and represent the definitive pool of follicles that determine the female reproductive lifespan (Zheng et al., 2014a). These two groups of follicles depend on two distinct populations of pre-granulosa cells. Recent studies have shown that granulosa cells of the central follicles differentiate from supporting cell precursors that are FOXL2-positive in the fetal ovary (Mork et al., 2012). In contrast, the authors suggested that granulosa cells of the adult cortical follicle derive from cells in the coelomic epithelium (Mork et al., 2012). In this study we have demonstrated that a second population of supporting cell precursors that express *Lgr5* exists in the cortical region of the fetal ovary. While at 4 weeks postnatally, activated medullary follicles of the first wave are still present (Zheng et al., 2014a), the fact that in the fetal ovary coelomic epithelial cells contribute to the first wave of granulosa cells until approximately 14.5 dpc (Mork et al., 2012) and that lineage-traced granulosa cells were also observed after exposure to tamoxifen at 17.5 dpc (Fig. 7) strongly suggests that *Lgr5*-positive cells in the fetal ovary give rise to granulosa cells of the second wave of follicles, that represent the definite pool of follicles.

We detected both LGR5 and FOXL2 in a few cells in the fetal ovary, however in each case the expression of one of the two factors was clearly dominant while the other was expressed at low levels. Based on both data presented here and previous observations (Mork et al., 2012) we posit that cells in and immediately below the ovarian surface epithelium express *Lgr5*. Through their proliferative action, these cells ingress into the ovary at which point they up-regulate *Foxl2* expression. Subsequently, FOXL2 down-regulates *Lgr5* expression and the cells differentiate into FOXL2-positive, *Lgr5*-negative granulosa cells. FOXL2 has been shown to directly transcriptionally repress other genes in the developing gonads (Pisarska et al., 2004; Takasawa et al., 2014; Uhlenhaut et al., 2009), however whether FOXL2 also directly inhibits *Lgr5* expression in the fetal ovary is yet to be shown.

Lgr5 plays a role in germ cell differentiation and survival

LGR5 binds R-spondin proteins with high affinity and thereby enhances RSPO/WNT/β-catenin signaling (Carmon et al., 2011; Chen et al., 2013; de Lau et al., 2011; Ruffner et al., 2012). The important roles played by RSPO1, WNT4 and β-catenin in mammalian gonad development and especially ovarian differentiation have been shown through the identification of mutations and deletions in human patients with disorders of sex development (Biason-Lauber et al., 2004; Mandel et al., 2008; Parma et al., 2006; Philibert et al., 2011), as well as gain- and loss-of-function analyses in mice (Chang et al., 2008; Chassot et al., 2008;

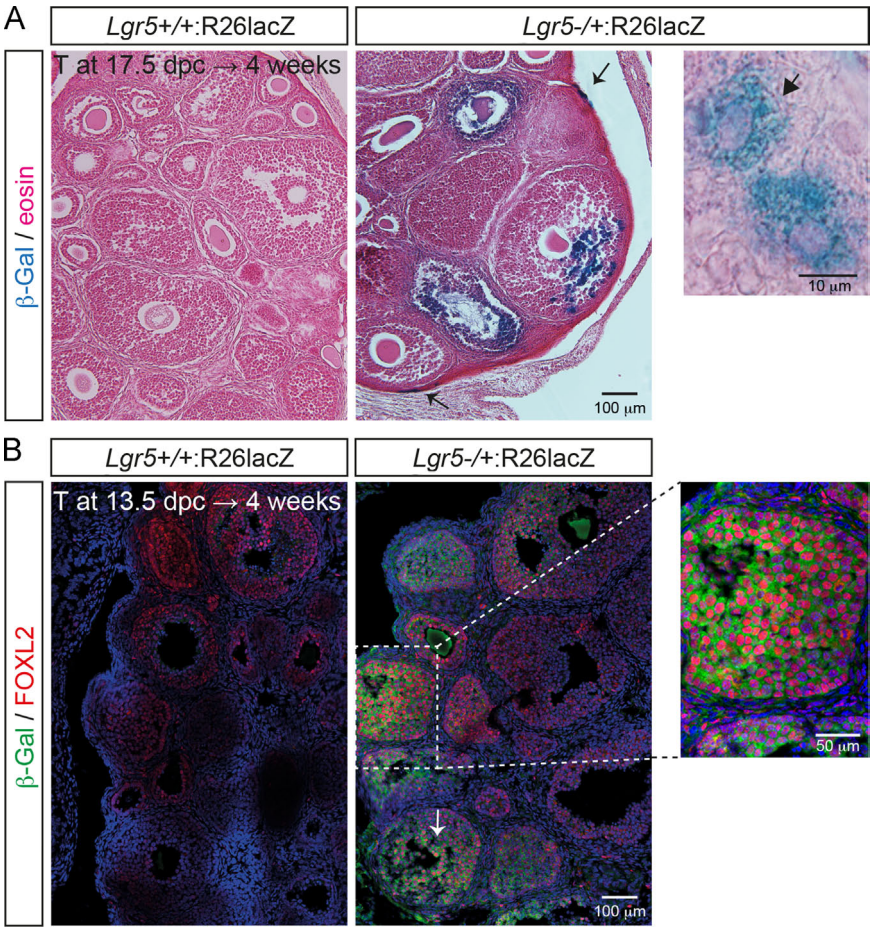

Fig. 7. *Lgr5*-positive cells in the foetal ovary differentiate into cortical granulosa cells. Pregnant females from $Lgr5^{+/+}$ or $Lgr5^{-/+}$ x R26LacZ matings were injected with tamoxifen at (A) 17.5 dpc or (B) 13.5 dpc and ovaries from 4-week-old offspring analyzed for β-galactosidase expression by (A) lacZ staining or (B) co-IF for β-galactosidase (green, marked by white arrows in bottom panel) and the granulosa cell marker FOXL2 (red) demonstrating that *Lgr5*-positive cells at both stages give rise to cortical granulosa cells. Black arrows in (A) indicate lacZ-positive ovarian epithelial cells (middle panel) and higher magnification of cortical follicles (right panel). The area marked in (B) by a rectangle is shown in higher magnification on the right.

Jeays-Ward et al., 2003; Kim et al., 2006; Maatouk et al., 2008; Vainio et al., 1999). The null phenotypes of *Wnt4* and *Rspo1* are very similar, including their propensity to undergo partial sex reversal with ectopic endothelial cell migration and coelomic vessel formation, impaired migration of steroidogenic cells and impaired germ cell proliferation, survival and entry into meiosis (Chassot et al., 2008; Jeays-Ward et al., 2003; Maatouk et al., 2013; Tomizuka et al., 2008; Vainio et al., 1999). The effects of loss of *Wnt4* or *Rspo1* on ovarian germ cells differ depending on the genetic background. While in $Wnt4^{-/-}$ ovaries germ cells are lost through increased apoptosis (Vainio et al., 1999), proliferation is inhibited with or without an increased germ cell apoptosis in $Rspo1^{-/-}$ ovaries (Chassot et al., 2011; Tomizuka et al., 2008). In contrast, loss of *Lgr5* in the fetal ovary led to a delay in germ cell differentiation. At 16.5 dpc no nuclear expression of the pluripotency marker OCT4 was detected. Higher magnification showed cytoplasmic staining. Cytoplasmic localization has been described for an OCT4 variant, OCT4B (Atlasi et al., 2008; Lee et al., 2006). However, the antibody we used (Santa Cruz, mouse monoclonal anti-OCT3/4 (C-10), sc-5279) should only detect the OCT4A isoform, suggesting that a more likely explanation is that the observed cytoplasmic staining is background due to the antibody used. We did not detect any CYP11A1-positive cells indicative of steroidogenic cell migration defect or the formation of a coelomic vessel in $Lgr5^{-/-}$ ovaries (data not shown). We suggest that this different phenotype is due to other genes that might contribute to, or replace, the activity of LGR5 by enhancing WNT/RSPO-signaling, while WNT4 and RSPO1 loss is not compensated for. In support of this view we showed that in addition to *Lgr5*, *Lgr4* is also expressed in ovarian somatic cells providing a plausible mechanism for genetic compensation in this pathway. Expression of *Lgr4* was also observed in supporting cells using microarray analysis of cells sorted from embryonic gonads (Jameson et al., 2012), in accordance with our observations. Moreover, *Lgr4* and *Lgr5* have been shown to functionally redundant in other systems (de Lau et al., 2011; Kinzel et al., 2014), suggesting that these two genes also have overlapping roles in the developing ovary.

Taken together, our data demonstrate a direct role for *Lgr5* in the development of the supporting cell lineage in the mammalian ovary and provide the first marker for the cortical granulosa cell precursors that establish the follicle reserve. In addition, we show that the fetal mouse ovary contains at least three different somatic cell types, including precursors for medullary and cortical granulosa cells and a third cell lineage, possibly representing pre-theca cells. These cell types are marked by the expression of *Foxl2*, *Lgr5*, and *Nr2f2*, respectively. Our findings provide important new insights into early lineage allocation and differentiation in the developing ovary. The identification of cell-type specific markers provides fundamental new tools for the tracking of these lineages to investigate their contribution to normal and pathological development in the mammalian ovary.

Author contributions

DW, MC, and RGR developed the concepts; RR, PB, JSP, AC, HC, PW and DW performed experiments; and all authors were involved in the analysis of data as well as preparing and editing the manuscript prior to submission.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.08.013.

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