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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.