Development of mammalian ovary

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Abstract

Pre-natal and early post-natal ovarian development has become a field of increasing importance over recent years. The full effects of perturbations of ovarian development on adult fertility, through environmental changes or genetic anomalies, are only now being truly appreciated. Mitigation of these perturbations requires an understanding of the processes involved in the development of the ovary. Herein, we review some recent findings from mice, sheep, and cattle on the key events involved in ovarian development. We discuss the key process of germ cell migration, ovigerous cord formation, meiosis, and follicle formation and activation. We also review the key contributions of mesonephric cells to ovarian development and propose roles for these cells. Finally, we discuss polycystic ovary syndrome, premature ovarian failure, and pre-natal undernutrition; three key areas in which perturbations to ovarian development appear to have major effects on post-natal fertility.

Key Words
- ovary
- fetus
- development
- polycystic ovary syndrome
- premature ovary failure

Journal of Endocrinology
(2014) **221**, R145–R161

Journal of Endocrinology

Introduction

The developmental origins of health and disease are an area of increasing research. The concept that pre- or perinatal alterations in environment can lead to permanent alterations in the structure and function of a number of organs has important implications for human health and the livestock industry. With respect to the ovary, pre-natal exposure to contaminants has been implicated in human polycystic ovary syndrome (PCOS), the leading cause of anovulatory infertility in women, as well as premature ovarian failure (POF) (Goswami & Conway 2005). In addition, gestational nutrition and infection are thought to have direct effects on the ovary, leading to impaired post-natal fertility; an important issue in domestic livestock. In understanding, how prenatal and perinatal factors can lead to impaired post-natal fertility, our understanding of ovarian development assumes increasing importance. This review aims to provide a summary of the current knowledge of ovarian development, bringing together the commonly accepted concepts as well as providing new insights and ideas. We discuss how changes in the pre-natal environment may alter ovarian development and lead to subsequent impaired fertility. Studies in sheep, cattle, and rodents will be the focus of the review. Figures 1, 2, and 3 present a schematic overview of the key events in ovarian development.

The beginnings of ovarian development

While differences in the timing and sequence of developmental events are apparent between mammalian species, ovaries of different mammalian species largely develop in a similar manner. The ovary is first apparent as a thickening of the coelomic epithelium on the medial aspect of the mesonephros, around day 22 in sheep, day 10.5 in mouse, and day 30 in cattle (Fig. 4A and B).

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DOI: 10.1530/JOE-14-0062

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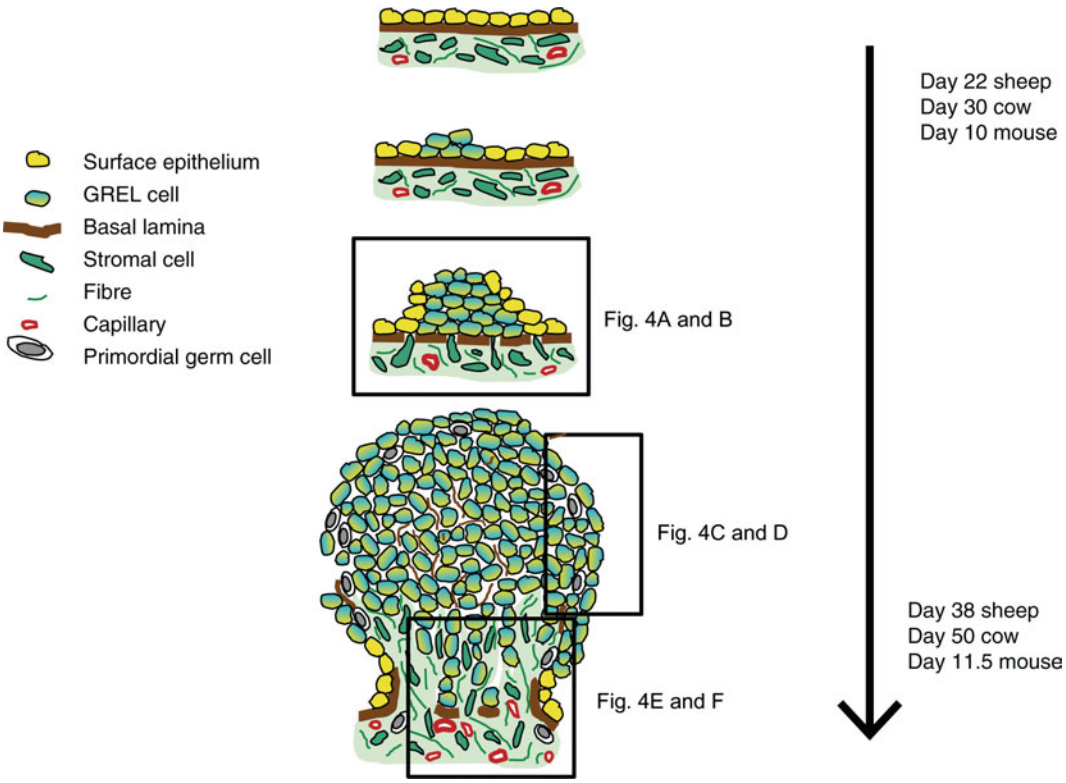

Figure 1
Schematic illustration of early ovarian development. Embryonic days 22–38 for sheep, days 30–50 for cattle, and days 10–11.5 for mice. Ovarian development begins with differentiation of gonadal ridge epithelial-like (GREL) cells from mesonephric epithelial cells and proliferation of these cells. Breakdown of the basal lamina underlying the mesonephric surface epithelium allows migration of stromal cells, vasculature, and oogonia into the ovary. Both GREL cells and oogonia continue to proliferate. Close associations between oogonia and GREL cells are formed through desmosome-like structures and patches of basal lamina appear at the periphery of the oogonia–GREL cell complexes. While the specific gene expression profile associated with GREL cells has yet to be demonstrated in sheep and mice, similarities in morphology between cattle, sheep, and mice would suggest their probable presence in all three species. Modified and reprinted with permission from Hummitzsch K, Irving-Rodgers HF, Hatzirodos N, Bonner W, Sabatier L, Reinhardt DP, Sado Y, Ninomiya Y, Wilhelm D & Rodgers RJ 2013 A new model of development of the mammalian ovary and follicles. *PLoS ONE* 2013 **8** e55578.

The mesonephros is a transient fetal kidney in mammals, composed of an array of glomeruli and collecting ducts located ventrally, as well as excretory ducts located dorsal to these. Throughout its development and subsequent regression, the mesonephros contributes tubules and cells to the developing ovary (Zamboni *et al*. 1979, Wartenburg 1982). What remains unclear is how early the mesonephros contributes cells to the developing ovary. While Zamboni *et al*. (1979) reported mesonephric cell migration into the ovary before day 30 (i.e. before gonadal sexual differentiation) in sheep, more recent studies of mice have indicated that initial mesonephric cell migration is dependent on the expression of *sry* and does not occur around the time of sexual differentiation (Capel *et al*. 1999). Remnants of mesonephric glomeruli and collecting ducts are still apparent in peri-ovarian tissue until around day 95 of gestation in sheep (Smith 2012).

Primordial germ cell migration

Primordial germ cells (PGC) migrate from the endoderm of the yolk sac, through the developing hindgut and then along the dorsal mesentery to the developing gonads (Mintz 1957) from around days 7 to 11 in mouse (Anderson *et al*. 2000), days 17 to 21 in sheep (Ledda *et al*. 2010), and days 18 to 31 in cattle (Wrobel & Süss 1998). PGCs continue to proliferate during their migration, from an initial population of 10–100 in mice, reaching around 25 000 by day 13.5 (Byskov 1986). PGC proliferation continues for an extended period during gonadal development. Kit ligand (KITL) and its receptor KIT play a critical role in the survival of migrating germ cells (Wilhelm *et al*. 2007). In addition, extracellular matrix proteins, notably fibronectin, are thought to play an important role in the migration of PGCs (Ffrench-Constant *et al*. 1991, Anderson *et al*. 1999). The totipotency of the PGCs appears

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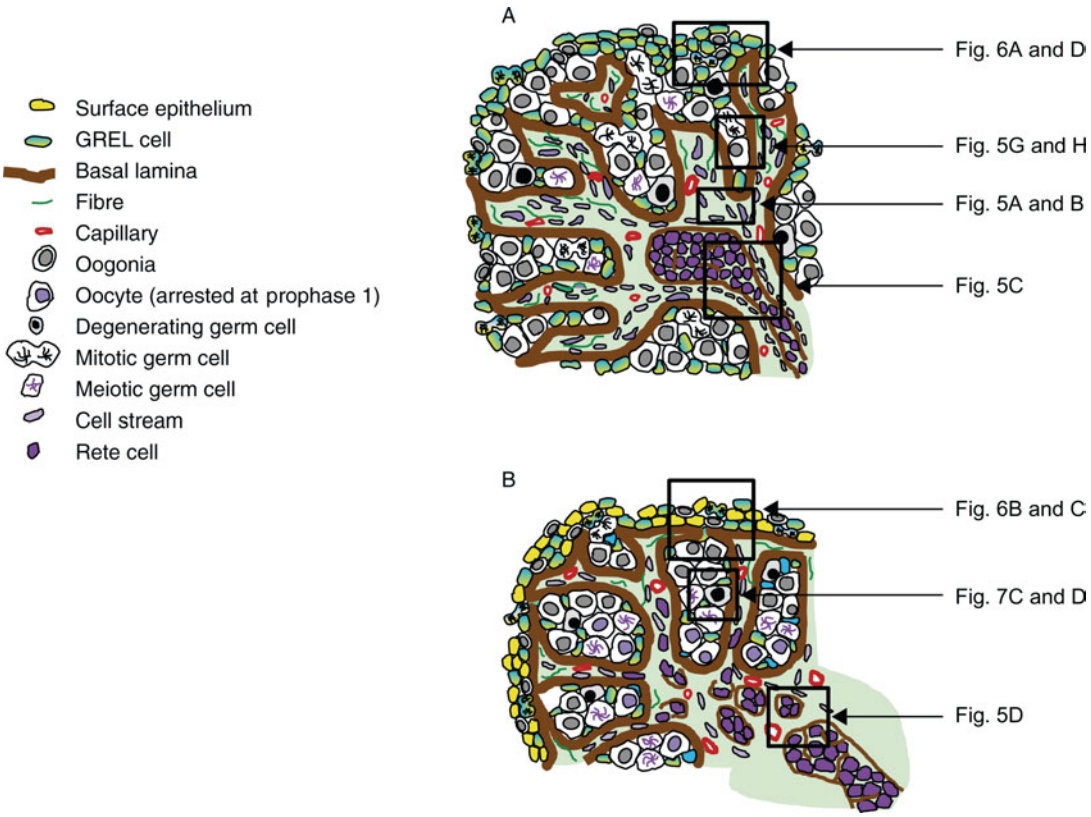

Figure 2
Schematic illustration of ovarian development during (A) ovigerous cord formation. Embryonic days 50–55 for sheep, days 70–100 for cattle, and days 12–15 for mice and (B) during ovigerous cord closure and surface epithelium development. Days 75–90 for sheep, days 100–130 for cattle, and days 15–17.5 for mice. Ovigerous cords develop further, enclosing germ cell–GREL cell complexes. The basal lamina isolates these complexes from the ovarian stroma but these complexes remain open at the periphery of the ovary. This allows the proliferating GREL cells at the ovarian surface to continue to make contact with proliferating germ cells, which increasingly become confined to the outer regions of the cortex. Germ cells entering meiosis and undergoing atresia become increasingly evident within the cords and GREL cells begin to differentiate into granulosa cells. Mesonephric-derived cell streams and subsequently rete tubules initially enter the ovary medulla but increasingly penetrate the ovarian cortex, further isolating the ovigerous cords from each other. The basal lamina beneath the ovarian surface progressively develops, isolating the ovigerous cords from the ovarian surface. Modified and reprinted with permission from Hummitzsch K, Irving-Rodgers HF, Hatzirodos N, Bonner W, Sabatier L, Reinhardt DP, Sado Y, Ninomiya Y, Wilhelm D & Rodgers RJ 2013 A new model of development of the mammalian ovary and follicles. *PLoS ONE* 2013 **8** e55578.

to be maintained by the expression of factors such as the nuclear transcription factor OCT4. Expression of OCT4 (*POU5F1*) continues in germ cells upon arrival at the gonad. OCT4 expression subsequently ceases in germ cells in an ovary at around day 13 in mice when meiosis is initiated (Pesce *et al*. 1998).

Development associated with sexual differentiation

While sex-specific gene expression occurs earlier, gonadal sex differentiation is considered to occur at day 12 in mice (Menke *et al*. 2003), day 32 in sheep (McNatty *et al*. 1995), and day 40 in cattle (Erickson 1966). Around the time of gonadal sexual differentiation, ovarian development is widely considered a less active pathway than testicular development. This view is based on the premise that ovarian development is the default pathway, demonstrated by Jost (1947), while testicular development is dependent on expression of the *SRY* gene. In addition, there are rapid morphological changes in the testis, which become apparent by the development of the testis cords and the tunica albuginea as early as day 32 in sheep and day 12 in mice. In contrast, in the ovary, ovigerous cord formation is not apparent until at least day 40 in sheep. At sexual differentiation, the ovary consists of at least five cell types, the ovarian surface epithelium; endothelial cells forming blood vessels; mesenchymal or stromal cells; pre-granulosa cells; and PGCs. Recent studies of bovine ovarian development have identified a population of

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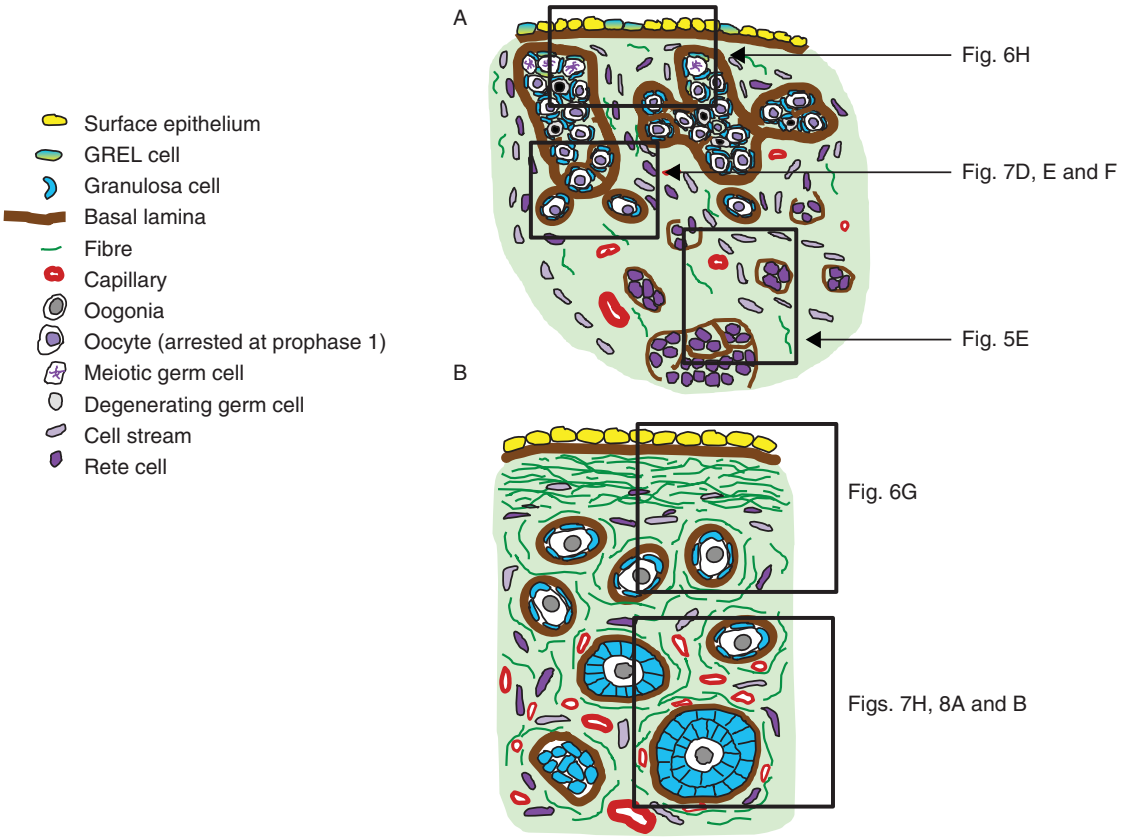

Figure 3
Schematic illustration of ovarian development during (A) follicle formation. Embryonic days 75–100 for sheep, days 130–210 for cattle, embryonic day 18 through post-natal day 8 for mice and (B) at completion of follicle formation day 100+ for sheep, day 210+ for cattle, post-natal day 8+ for mice. The ovarian surface becomes completely isolated from ovigerous cords and stromal cells by the completion of a continuous sub-epithelial basal lamina, GREL cells at the ovarian surface differentiate into mature surface epithelial cells, while the GREL cells within the cords complete their differentiation into granulosa cells. Within the cords germ cell meiosis is arrested at the completion of prophase 1. Atresia of germ cells results in the loss of most germ cells; however, the associated GREL/granulosa cells are 'reassigned' to the remaining germ cells. Continuing production of basal lamina within the cords, between individual germ cell–granulosa cell complexes results in the formation of individual follicles, a process augmented by the continuing penetration of cell streams and rete cells into the ovarian cortex. Most germ cells (other than a small number of 'lost germ cells' in the ovarian medulla or a few trapped in the surface epithelium) appear in follicles. Activation of follicles can appear simultaneously with release from ovigerous cords. Modified and reprinted with permission from Hummitzsch K, Irving-Rodgers HF, Hatzirodos N, Bonner W, Sabatier L, Reinhardt DP, Sado Y, Ninomiya Y, Wilhelm D & Rodgers RJ 2013 A new model of development of the mammalian ovary and follicles. *PLoS ONE* 2013 **8** e55578.

cells termed gonadal ridge epithelial-like cells (GREL), which form the genital ridge from the surface epithelium of the mesonephros. GREL cells have a distinctive gene expression profile, including the expression of cytokeratin 19, plakophilin-2, and desmoglein-2 (Hummitzsch *et al.* 2013). Using this distinctive gene expression profile, this study demonstrated that GREL cells give rise to the ovarian epithelium. Furthermore, the study showed that it is likely that all pre-granulosa cells are derived from GREL cells (Hummitzsch *et al.* 2013). Following gonadal sexual differentiation, and before ovigerous cord formation, it is GREL cells which make initial contact with germ cells and establish the first germ cell–pre-granulosa cell complexes (Fig. 4C and D).

After ovigerous cord formation, it is the GREL cells, located near or at the ovarian surface, which then contribute pre-granulosa cells to the increasing population of germ cells. While the specific GREL cell expression profile has yet to be examined in sheep and mice, similar morphological descriptions of the events associated with GREL cells would indicate that GREL cells exist in these species.

Ultrastructurally, both oogonia and pre-granulosa cells are distinct, in that they contain lipid droplets and elements of smooth endoplasmic reticulum, i.e. characteristics of steroid-producing cells. *In vitro*, fetal sheep ovaries from as early as day 28 of gestation have the capacity to produce progesterone, androstenedione, and

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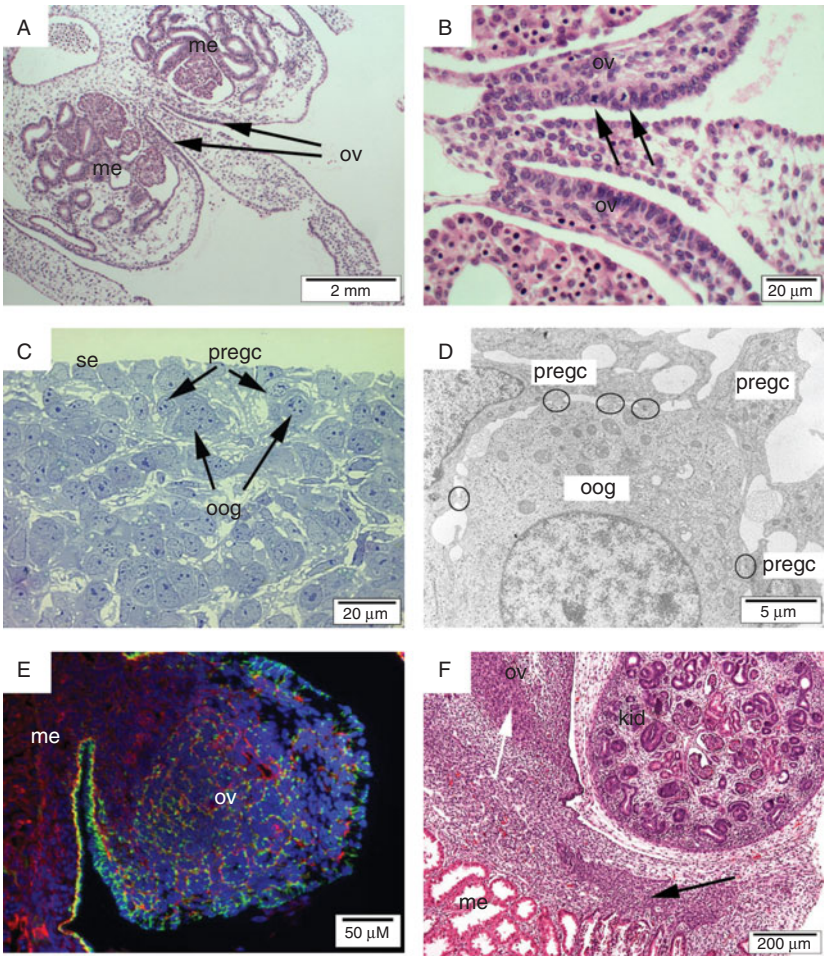

Figure 4
(A) Embryonic day 24 sheep ovary. Ovaries (ov) developing from the epithelium of the mesonephros (me). (B) Embryonic day 24 sheep. Differentiation and proliferation of mesonephric surface epithelium (se) into developing ovary (ov). Note mitotic figures (arrows). (C) Embryonic day 38 sheep 1 micron section showing both surface epithelium (se) with no underlying basement membrane and initial contact between oogonia (oog) and pre-granulosa/GREL cells (pregc). (D) Embryonic day 38 sheep, electron micrograph showing initial contact as desmosomes (circled) between oogonia (oog) and pre-granulosa cells (pregc). (E) Embryonic day 47 cattle, laminin 111 red, CK19 green, DAPI blue, illustrating cell continuity between the mesonephros (me) and ovary (ov). Also note defined surface epithelium at the base of the ovary with strong staining for CK19 and laminin 111 staining the underlying epithelium illustrating the presence of a basal lamina which is restricted to the base of the ovary. Beginnings of cord formation observed by laminin 111 staining throughout the ovary. (F) Embryonic day 35 sheep. Ovary (ov), mesonephros (me) and kidney (kid). Cells from regressing glomeruli accumulating at the base of the mesonephros (black arrow). Examination of serial sections shows continuity between these cells and cells penetrating the ovarian medulla (white arrow). E was modified and reprinted with permission from Hummitzsch K, Irving-Rodgers HF, Hatzirodos N, Bonner W, Sabatier L, Reinhardt DP, Sado Y, Ninomiya Y, Wilhelm D & Rodgers RJ 2013 A new model of development of the mammalian ovary and follicles. *PLoS ONE* 2013 **8** e55578. C and D were modified and reprinted with permission from Sawyer HR, Smith P, Heath DA, Juengel JL, Wakefield SJ & McNatty KP 2002 Formation of ovarian follicles during fetal development in sheep. *Biology of Reproduction* **66** 1134–1150.

estradiol. *In vivo*, ovarian steroid contents are elevated from day 35 of gestation (Lun *et al.* 1998). *In situ* hybridization studies showed that at this stage the steroid-producing cells are concentrated at the cortical-medullary boundary (Quirke *et al.* 2001). Collectively, these studies indicated that the early oogonia–pre-granulosa cell complexes are the major sites of early steroid synthesis in the ovary.

Migration of mesonephric cells

Migration of mesonephric cells into the developing gonad and their proliferation is a well-documented phenomenon (Smith 2012). Studies in mice indicated that migration of mesonephric cells into the testis, but not the ovary, occurs before gonadal sexual differentiation (Capel *et al.* 1999). However, these migrating cells are

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exclusively endothelial cells, which form the testis-specific vasculature (Jeays-Ward *et al*. 2003, Combes *et al*. 2009). In contrast in sheep, mesenchymal cells of the giant nephron are thought to migrate into the ovary before sexual differentiation (Zamboni *et al*. 1979), a phenomenon also observed in cattle (Kenngott *et al*. 2013). In addition, in cattle the presence of mesonephric-tissue-derived GREL cells also appears to precede sexual differentiation. Roles for other mesonephric cell types remain unclear. Migration of mesonephric cells appears to follow a well-defined pattern. In sheep, from around day 24 a loose arrangement of mesenchymal cells has been reported to enter the ovary (Juengel *et al*. 2002). Based on morphological criteria, it was postulated that these made early contact with germ cells around the time of sexual differentiation. As such, these cells would therefore correspond to the GREL cells reported in cattle. From around the time of sexual differentiation, cells from regressing glomeruli are observed entering the ovary (Fig. 4E and F). A pronounced continuum of cells between the mesonephros and ovary is apparent from around day 38 in sheep. This mass of cells extends into the medulla of the ovary from where branches are observed extending further into the ovarian cortex. These cells are irregular in shape and exhibit pseudopodia, a characteristic of migrating cells. In particular, as they traverse the ovary, these cells are easily distinguishable at the light microscopic level as they appear ordered and are orientated in the same direction and consequently the term 'cell streams' has been used to describe them (McNatty *et al*. 2000; Fig. 5A). As the glomeruli and ducts of the mesonephros further regress, basement membranes become increasingly evident in the cell continuum connecting the ovary and regressing mesonephros, such that by day 55 in sheep, virtually all migrating cells are enclosed within a defined basement membrane (Fig. 5B). Extending from the remnants of the mesonephros to the ovarian medulla, this membrane-bound continuum is thought to represent the extra-ovarian and connecting rete. Located in the ovarian medulla, the connecting rete continues to increase in size through both migration and cell proliferation, reaching its maximum size at day 95 in sheep (Smith 2012; Fig. 5C and D). Increasingly, from day 55, branches of the connecting rete become apparent, which disconnect from the main body and appear to 'float' within the ovarian medulla. Once isolated from the connecting rete, these branches are thought to represent the intra-ovarian rete (Fig. 5C, D and E). Predominantly, at the medulla–cortex interface, the basement membrane, which encloses the intra-ovarian rete, becomes discontinuous and the cells 'spill over' and subsequently populate the ovarian cortex (Fig. 5D and E). The cells of the connecting and intra-ovarian rete show consistent expression of *WT1* and *FST* during development. In sheep, expression of *ESR1, ESR2,* and *AR* is evident from day 55, and *PDGFA* from day 75, while at the same stage *CYP19*A expression is evident in those cells 'spilled' from compromised intra-ovarian tubules (Juengel *et al*. 2006, Smith 2012). Between day 40 and at least day 75, expression of steroidogenic enzymes such as *CYP11A1, CYP19A1, CYP17A1,* and *STAR* is increasingly localized to the cell streams, which also express *ESR1*, *ESR2,* and *AR* (Juengel *et al*. 2006, Garverick *et al*. 2010). From mid-gestation, proliferative activity of cortical stromal cells within the ovary remains relatively low (Fig. 5F), while the cortical volume increases from 1.5 mm^3 at day 55 to 3.8 mm^3 at day 90 and 6.4 mm^3 at day 135. The morphological evidence is indicative of the ovarian cortex containing a significant proportion of cells derived from the migration of both the intra-ovarian rete cells and the cells of the cell streams. It is tempting to suggest that either of these cell types may be the progenitors of thecal cells later in development. This concept is not without support. In newborn mice, Honda *et al*. (2007) isolated a population of putative thecal stem cells. Hatzirodos *et al*. (2012) also provided evidence for clusters of putative thecal stem cells in the adult bovine ovary based on the localization of proteoglycans generally associated with stem cell niches. Further evidence comes from differences in the morphology of the rete ovarii in a sheep model for PCOS (Smith *et al*. 2009). Some previous studies have concluded that the rete is the source of pre-granulosa cells (Peters & Pedersen 1967, Byskov & Lintern-Moore 1973). However, it appears that given the inaccessible nature of the ovigerous cords during the critical period of development that this is unlikely to be the case. This is not to preclude the possibility that the rete has paracrine signaling roles in cord/follicle development, as has been implicated for its role in the initiation of meiosis (Byskov 1975, Wai-Sum & Baker 1976).

Ovigerous cord formation

Ovigerous cord formation is characterized by the simultaneous occurrence of three critical events. Firstly, increasing patches of basal lamina material become apparent at the outer margins of oogonia–pre-granulosa cell complexes (Fig. 5G and H). Secondly, pre-granulosa cells develop cytoplasmic extensions, which progressively isolate oogonia from each other (Sawyer *et al*. 2002). Finally, there is an increasing infiltration of medullary

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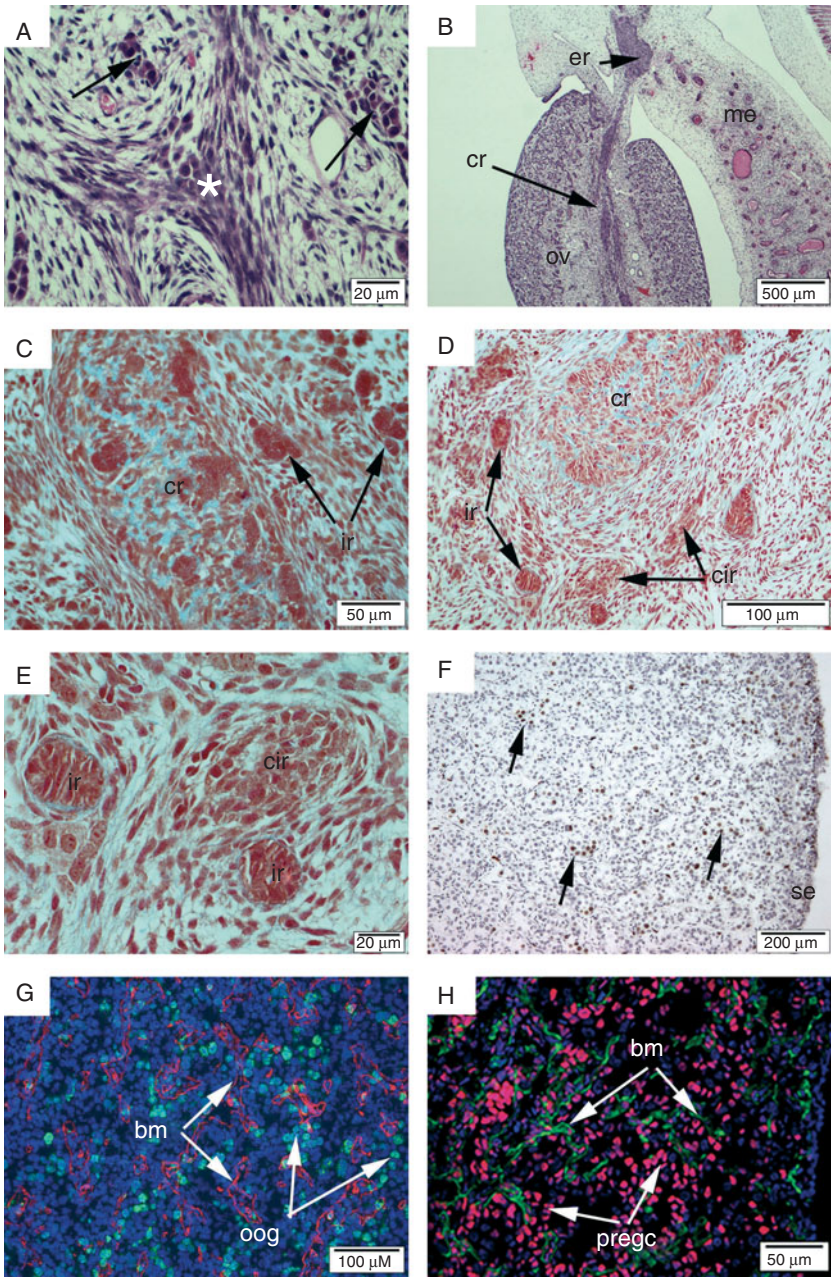

Figure 5

(A) Embryonic day 55 sheep ovary. Medullary region showing example of a mesonephric derived cell stream (white *). Cells are elongated and orientated in a similar direction. Note also clusters of 'lost oocytes' (black arrows). (B) Embryonic day 55 sheep ovary showing rete connection between ovary (ov) and mesonephros (me). er, extraovarian rete; cr, connecting rete. (C) Embryonic day 75 sheep ovary, connective tissue stain. Medullary region illustrating the discrete (membrane-bound) nature of connecting rete (cr) and intraovarian rete (ir). (D) Embryonic day 95 sheep ovary. Further development of connecting rete (cr) and intraovarian rete (ir). Note regions where the discrete nature of intraovarian rete becomes compromised (cir). These cells subsequently populate the ovarian cortex. (E) Embryonic day 95 sheep ovary. High power image showing difference between 'intact' intraovarian rete (ir) and intraovarian rete whose discrete nature has become compromised (cir). (F) Embryonic day 45 sheep ovary BrdU immunohistochemistry, illustrating proliferation is largely confined to oogonia (black arrows) and cells at the ovarian surface (se). (G) Embryonic day 88 cattle ovary. Collagen type IV red, OCT 3/4 green and DAPI blue. (H) Embryonic day 83 cattle ovary. FOXL2 red, perlecan green and DAPI blue. Both G and H show early development of ovigerous cords, as evidenced by development of basal membrane (bm: red in G and green in H), isolating oogonia (oog: green in G) and pre-granulosa cells (pregc: red in H) from ovarian stroma. G and H were reprinted with permission from Hummitzsch K, Irving-Rodgers HF, Hatzirodos N, Bonner W, Sabatier L, Reinhardt DP, Sado Y, Ninomiya Y, Wilhelm D & Rodgers RJ 2013 A new model of development of the mammalian ovary and follicles. *PLoS ONE* 2013 **8** e55578.

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stromal/rete cells between cortical complexes. The resultant cords are clusters of oogonia and pre-granulosa cells isolated from the ovarian stroma by a basal lamina (Fig. 6A, B and C). They extend radially through the increasingly well-defined ovarian cortex. Arguably, the extent and manner of cord formation varies between species and appears to be related to the timing of cord formation and/or the timing of meiosis relative to gonadal differentiation.

In species with delayed meiosis (an extended period between gonadal sexual differentiation and the onset of meiosis), the development of ovigerous cords can differ. In sheep, pigs, and cattle clearly defined ovigerous cords are formed almost simultaneously with the development of testicular cords, whereas in rabbits and humans the ovigerous cords are not so clearly defined. In rodents, for which the period between gonadal differentiation and meiosis is minimal, the formation of clear ovigerous cords was reported not to occur (Byskov 1986). However, more recent studies have referred to not only the presence of membrane-bound germ cell clusters and ovigerous cords in rodents (Ungewitter & Yao 2013), but also their role and importance in successful ovarian development (Nicholas *et al*. 2010, Kimura *et al*. 2011). The evidence increasingly supports the presence of membrane-enclosed clusters of germ cells and somatic cells in all mammalian fetal ovaries, although the timing and extent of their development may be the subject of some interspecies variation.

Two important characteristics of the ovigerous cords laid the foundation for understanding subsequent events related to follicle formation. While a basal lamina separates the ovigerous cords containing the pre-granulosa cells and oogonia/oocytes from the ovarian stroma, the ovigerous cords are open to the surface of the ovary (Fig. 6B and C). In addition, BrdU labeling has shown that while germ cells continue to proliferate within the ovigerous cords the pre-granulosa cells, identified by their close association with germ cells, are largely quiescent (Sawyer *et al*. 2002; Fig. 6D and E). In the developing sheep ovary, during the period from day 40, when ovigerous cord formation is well underway, through until day 75, germ cell numbers increase from approximately 50 000 to 805 000 (Smith *et al*. 1993). By counting the number of pre-granulosa cells associated with each germ cell, we estimate that in the same period pre-granulosa cell numbers increase from 200 000 to 4 250 000. Given that pre-granulosa cells are quiescent, and this increase in their number occurs almost exclusively while pre-granulosa–germ cell complexes are within the ovigerous cords, i.e. isolated from the ovarian stroma, pre-granulosa cells must therefore be recruited from the somatic cells at the periphery of the ovigerous cords. These cells may come from either the ovarian surface, or from those cells immediately underlying the surface, all of which are derived from GREL cells, at least in the bovine ovary (Hummitzsch *et al*. 2013). A similar source of pre-granulosa cells in the mouse ovary has been suggested (Mork *et al*. 2012). This study, while in general agreement with the concepts proposed here – concluding that most if not all granulosa cells are derived from the surface epithelium – adds another level of complexity to the issue. A comprehensive set of experiments suggest that, at least in mice, the surface epithelium contributes pre-granulosa cells in two distinct waves. The first wave occurs just before sexual differentiation and the second occurs immediately post birth as follicles are forming. Similarly, Harikae *et al*. (2013) also propose two waves of pre-granulosa cell recruitment in mouse. This study uses the premise that in the developing ovary only the precursors of pre-granulosa cells retain the ability to respond to forced SRY action and activate expression of *Sox9*. Two such populations of cells were noted, one associated with the epithelium and another located in the medulla adjacent to the mesonephros. The study demonstrates that the medullary population of cells contributes to the first wave of follicles, which grow in the pre-pubertal period, while the epithelial-associated cells contribute to follicles which grow predominantly in the post-pubertal period. The concept of two waves of pre-granulosa cell recruitment may be applicable to most mammalian species as Sawyer *et al*. (2002) described an initial attachment of pre-granulosa cells to oocytes shortly after sexual differentiation, but before ovigerous cord formation, followed by the majority of pre-granulosa cell recruitment following cord formation. Furthermore, this concept fits with the GREL cell hypothesis proposed by Hummitzsch *et al*. (2013) as these cells are initially noted at the ovarian mesonephric border before becoming increasingly localized to the ovarian epithelium. In mice, follicles containing granulosa cells recruited during the first wave are then recruited into the growing pool immediately after birth and before puberty. As such, these follicles are thought to be largely anovulatory (Eppig & Handel 2012). The anovulatory nature of these follicles may be due to the absence of a mature hormonal environment; however, functional differences in the follicle may also play a role. In a recent study in mice, Zheng *et al*. (2014) have not only shown functional differences between first- and second-wave follicles, but also challenged the concept that first-wave follicles are anovulatory. Using an inducible *Foxl2* knock-in model (induced at 16.5 days *post coitum*),

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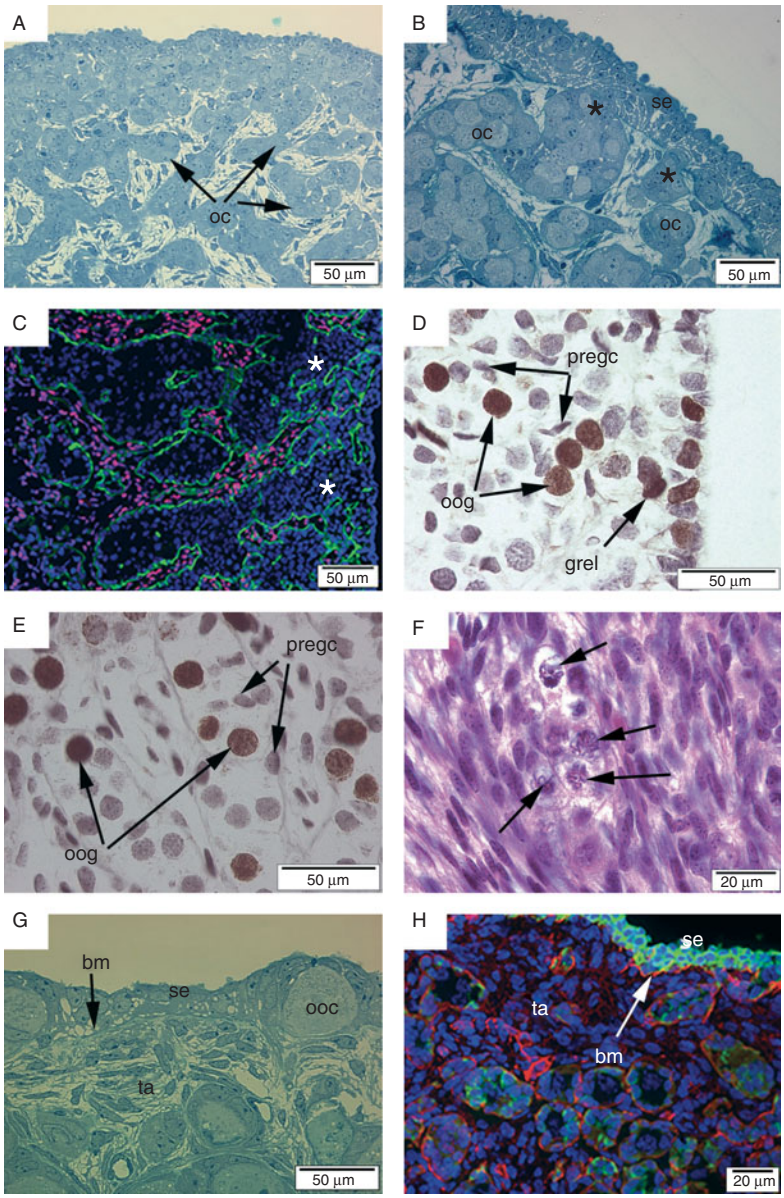

Figure 6

(A) Embryonic day 55 sheep ovary 1 µm section showing well-developed ovigerous cords (oc) extending radially through the ovarian cortex. (B) Embryonic day 75 sheep ovary 1 µm section showing open connections (black, *) between well-defined ovigerous cords (oc) and the ovarian surface (se). (C) Embryonic day 96 cattle ovary. COUP-TFII red, stromal cells; perlecan green, basement membrane and DAPI blue. Ovigerous cords are open to the ovarian surface as evidenced by discontinuous membranes below the ovarian surface (white, *). Stromal cells stained with COUP-TFII penetrate the ovarian cortex, continuing to separate ovigerous cords. (D) Embryonic day 75 sheep ovary BrdU immunohistochemistry at the ovarian periphery showing proliferation of germ cells (oog) and surface epithelial/GREL cells (grel) while pre-granulosa cells within cords are quiescent (pregc). (E) Embryonic day 75 sheep ovary BrdU immunohistochemistry near the cortical–medullary boundary showing proliferation of germ cells (oog) while pre-granulosa cells within cords are quiescent (pregc). (F) Embryonic day 75 sheep ovary. A cluster of 'lost germ cells', some at prophase I of meiosis (black arrows), located in the medulla of the ovary. Note the near absence of pre-granulosa cells and the absence of an enclosing basement membrane. (G) Embryonic day 90 sheep ovary. Formation of a mature surface epithelium (se) overlying a complete basement membrane (bm) and tunica albuginea (ta). Note oocyte 'trapped' within the surface epithelial layer (ooc). (H) Embryonic day 241 cattle. CK19 green, nidogen 2 red DAPI blue. Mature surface epithlium (se) expressing CK19 overlying a basement membrane (bm), containing nidogen 2, and a developing tunica albuginea (ta). C and H were reprinted with permission from Hummitzsch K, Irving-Rodgers HF, Hatzirodos N, Bonner W, Sabatier L, Reinhardt DP, Sado Y, Ninomiya Y, Wilhelm D & Rodgers RJ 2013 A new model of development of the mammalian ovary and follicles. *PLoS ONE* 2013 **8** e55578. D was modified and reprinted with permission from Sawyer HR, Smith P, Heath DA, Juengel JL, Wakefield SJ & McNatty KP 2002 Formation of ovarian follicles during fetal development in sheep. *Biology of Reproduction* 2002 **66** 1134–1150.

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this study could not only distinguish between first- and second-wave follicles but also trace their development. The study showed that first-wave follicles remained in the ovary up until three months of age, thereby contributing to not only the onset of puberty but also to fertility in the immediate post-puberty period. In addition, the study showed that first-wave follicles develop at approximately twice the rate of second-wave follicles, taking 23 days to reach the antral stage compared with 47 days for second-wave follicles. The combined evidence of many of these publications indicates that such a scenario is likely to occur in both sheep and cattle. If so, this has some important implications, particularly in the sheep industry where the mating of animals at puberty (8–10 months of age) is rarely adopted as the fertility of these animals is 50% less than that of those mated at 2 years of age (Kenyon *et al*. 2004). It is thought that oocyte competence plays a major role in this reduced fertility (Quirke & Hanrahan 1983). The concept of rapidly growing follicles releasing immature oocytes during an animal's initial oestrous cycles may go some way to explaining this issue and provide insights for overcoming the problem.

Throughout the period of cord and follicle formation, Sawyer *et al*. (2002) also reported the presence of isolated or small clusters of large cells in the ovarian medulla. Based on their morphology, ultrastructure, and the observation that they often appeared to have entered prophase of meiosis one, these cells were assumed to be germ cells, which had failed to be incorporated into ovigerous cords (Fig. 6F). Around the time of follicle formation, these 'lost germ cells' often became associated with elements of the ovarian rete, forming structures which resembled early growing follicles. The fate of these structures remains unclear.

During the later stages of follicle formation, once the stroma has penetrated between the ovigerous cords, reaching just below the GREL cells at the ovarian surface, the peripheral GREL cells differentiate to form the mature OSE (Hummitzsch *et al*. 2013) with an underlying basement membrane (Fig. 6G and H). This study contends that during the early stages of development. the ovary does not possess a defined surface epithelium (Fig. 7A). In support of this, both Garverick *et al*. (2010) and Kenngott *et al*. (2013) noted the lack of a well-formed epithelium in developing ovarian tissues in cattle, while McNatty *et al*. (2000) also reported a less-prominent epithelial layer following initial formation of the gonadal ridge. Further evidence supporting the lack of a mature ovarian surface epithelium comes from the observation that surface epithelial expression of *ESR1*, a characteristic of the ovarian surface epithelial layer is not noted until at least day 55 in sheep and day 75 in cattle (Garverick *et al*. 2010).

Meiosis, apoptosis, and follicle formation

Cell development within the ovigerous cords centers on three events: the initiation of germ cell meiosis, germ cell apoptosis, and follicle formation. Germ cell meiosis is first evident at day 52 in sheep (Mauleon & Mariana 1976), day 75 in cattle (Erickson 1966), and day 13 in mouse (Borum 1961). In sheep and cattle, meiosis starts in the innermost regions of the cortex, thereafter spreading throughout the cortex. Classical transplantation and microscopy studies in hamsters (Wai-Sum & Baker 1976) and mice (Byskov 1974, Byskov *et al*. 1977) have demonstrated the importance of the rete ovarii in both the initiation of meiosis and follicle formation in these species. In these studies, sections of the fetal ovary rich in rete were removed and cultured or grafted into nude mice hosts, which resulted in severe inhibition of meiosis and follicle formation. However, the conclusion that it is rete cells that affect both meiosis and follicle formation does not exclude the possibility that cells of the 'cell streams' may influence these events as these are also concentrated in areas rich in ovarian rete. A number of studies have identified retinoic acid as a key factor in controlling the initiation of meiosis (Koubova *et al*. 2006, Li & Clagett-Dame 2009). In an elegant study, Bowles *et al*. (2006) provides strong evidence indicating that in mouse translocation of mesonephric-derived retinoic acid to the ovary drives the initiation of meiosis. In contrast to this study, Le Bouffant *et al*. (2010) showed that in humans ovarian-produced retinoic acid is required for the initiation of meiosis. In a rather contentious study Kumar *et al*. (2011) contended that retinoic acid is not required for the initiation of meiosis. As retinoic acid synthesis is largely controlled by the expression of retinaldehyde dehydrogenase-2 (*Raldh2*) (*Aldh1a2*), this study used *Raldh2*$^{-/-}$ as well as *Raldh2*$^{-/-}$ and *Raldh3*$^{-/-}$ double mutant mice to show that induction of ovarian *Stra8*, a pre-meiotic gene required for the initiation of meiosis, occurs in the absence of detectable levels of retinoic acid. Expression of *Scp3*, a gene expressed during meiotic prophase, was also unaffected by the absence of retinoic acid and both *Raldh2*$^{-/-}$ and WT germ cells appeared to be engaged in meiotic recombination. Furthermore, in the testis expression of *Cyp26b1* is thought to degrade retinoic acid, thereby preventing retinoic acid-induced meiosis in the testes. However, inhibition of *Cyp26b1* in *Raldh2*$^{-/-}$ testes allowed the induction of *Stra8*, despite the absence of retinoic acid.

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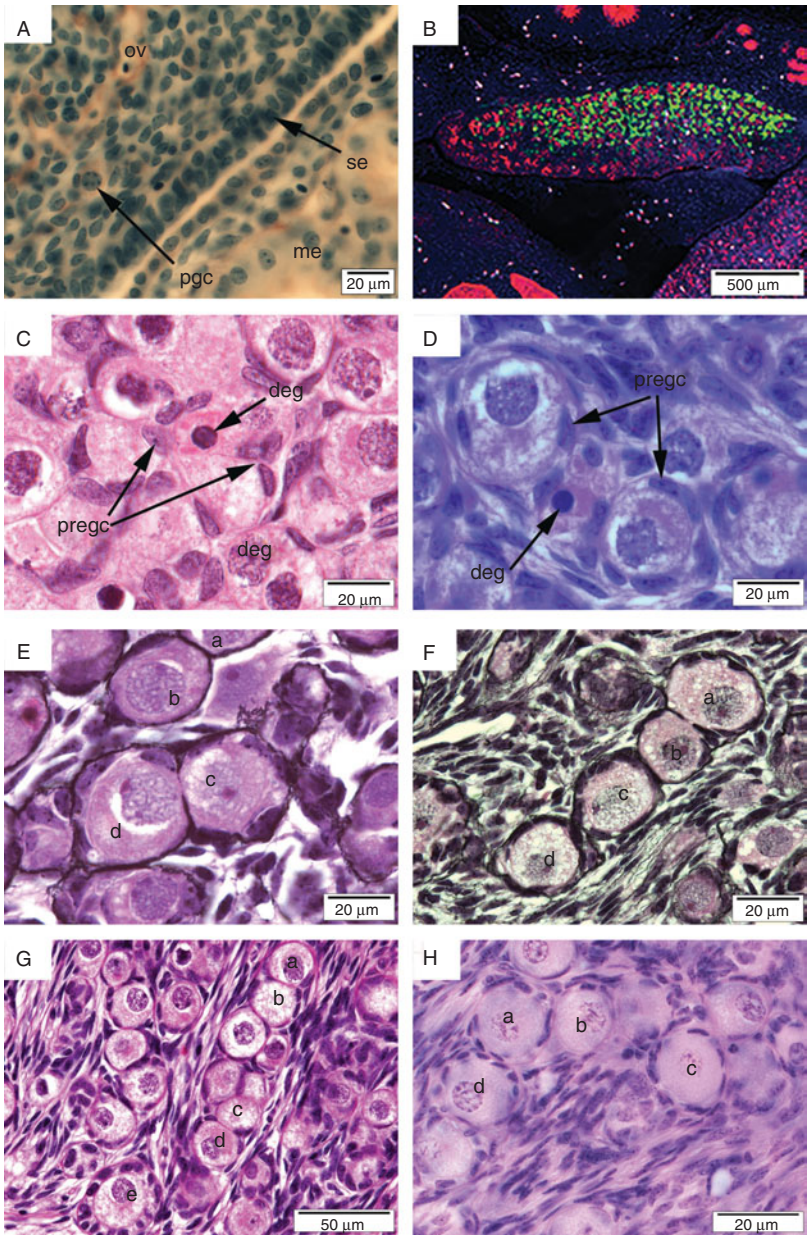

Figure 7

(A) Embryonic day 28 sheep ovary alkaline phosphatase. While cells of the ovarian surface (se), adjacent to the mesonephros (me), are morphologically well defined they lack an underlying basement membrane. Note migrating primordial germ cell (pgc). (B) Day 13.5 mouse ovary. FOXL2 is stained green E- cadherin red. FOXL2-positive cells (putative pre-granulosa or GREL cells) show cell/follicle development progressing in an anterior to posterior (left to right) direction. (C) Embryonic day 75 sheep ovary. Germ cell atresia during early follicle formation. Degenerating germ cells (deg) exhibiting classical morphology associated with apoptosis – nuclear condensation and cell shrinkage. Note that pre-granulosa cells (pregc) associated with degenerating germ cells remain healthy. (D) Embryonic day 90 sheep ovary. Germ cell atresia during late follicle formation follows a similar pattern with degenerating germ cells (deg) exhibiting apoptotic morphology and associated pre-granulosa cells (pregc) remain healthy. (E) Embryonic day 90 sheep ovary periodic acid methionine Schiff stain. Progressive separation of developing follicles. Follicles a and b are completely separated by a basement membrane whereas follicles c and d are not yet completely separated. (F) Embryonic day 90 sheep ovary, periodic acid methionine Schiff stain. Progressive separation of developing follicles. Follicles a and b are incompletely separated by basement membrane, follicle b and c are completely separated by basement membrane, follicles c and d are completely separated by basement membrane and are beginning to physically separate. (G) Embryonic day 90 sheep ovary. Variation in (pre-) granulosa cell number in forming and newly formed follicles. Follicle a=1 pregc, follicle b=3 pregc, follicle c=1 pregc, follicle d=5 pregc, and follicle e=15 pregc. Note that follicle e contains cuboidal granulosa cells and therefore is likely to be activated. (H) Embryonic day 90 sheep ovary. Variation in (pre)granulosa cell number in forming newly formed follicles. Follicle a=5 pregc, follicle b=2 pregc, follicle c=7 pregc, and follicle d=7 pregc.

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The results provide a seemingly strong case for the conclusion that meiosis occurs independently of retinoic acid and that *Cyp26b1* controls meiosis in the testes by metabolising a substrate other than retinoic acid. The study of Kumar *et al*. (2011) raises many questions but also has limitations, many of which are highlighted in a recent review (Griswold *et al*. 2012). It appears the body of evidence still supports retinoic acid as a key player in the initiation of meiosis while not precluding the involvement of as yet unknown factors.

The importance of mesonephric-derived retinoic acid in mice compared with ovarian-produced retinoic acid in humans highlights further differences between rodents and humans. It seems likely that these differences extend to other larger mammals when compared with rodents. While in sheep and cattle, similar to humans, meiosis and follicle development progresses from the inner to the outer regions of the cortex, in rodents these events progress in an anterior to posterior direction (Fig. 7B). This may signal species differences either in the site of retinoic acid production (mesonephric in rodents, and ovarian in humans and larger animals) or in the timing and organization of mesonephric cell penetration of the cortex. While arguably in rodents, the rete ovarii has been implicated as having a role in the initiation of meiosis, in sheep and cattle during the period of meiosis it is the 'cell streams' that progressively penetrate most of the ovarian cortex in parallel with the appearance of meiosis progressing outwardly, while the intra-ovarian rete remains predominantly a medullary component, still largely confined within a basement membrane. A major point of contention between studies in smaller mammals (Byskov 1975) and more recent studies in sheep and cattle (Juengel *et al*. 2002, Garverick *et al*. 2010) is the presence of open connections between ovigerous cords and other ovarian cell types, with these being reportedly present in mice, cats, minks, and ferrets, but absent in both sheep and cattle. Collectively, these studies indicate that while similar factors and signaling mechanisms may be involved in the initiation of meiosis, the production and/or delivery of these factors is the subject of species differences between rodents and larger mammals.

At the same time that germ cells enter meiosis, the first significant signs of germ cell death are also observed. The rate of germ cell death increases while the proliferation rate decreases, such that the number of germ cells present decreases by 80% between days 75 and 90 in sheep (Smith *et al*. 1993), and days 130 and 170 in cattle (Erickson 1966). This precipitous drop in germ cell number is a feature of most, if not all, mammalian species during ovarian development. A popular hypothesis suggests that the underlying causes of germ cell death are related to either the failure of meiosis or poor follicle formation, which could be due to insufficient pre-granulosa cells. The period of major germ cell death in sheep (Smith *et al*. 1993) and cattle (Erickson 1966) spans the developmental period when meiosis and follicle formation take place. In mice, two waves of germ cell death have been reported, one during the period of meiosis at day 13.5–15.5 and in a second-wave during follicle formation at day 17.5 to postnatal day 1 (Sarraj & Drummond 2012). Those germ cells undergoing cell death exhibit the classic characteristics of apoptosis: nuclear condensation, cell shrinkage, and fragmentation (Fig. 7C and D) as well as DNA laddering with positive TUNEL staining. There are numerous studies showing that germ cell apoptosis, currently widely accepted as the major mechanism of germ cell death, is mediated through the BCL2 family of proteins (for review see Aitken *et al*. (2011)). However, more recent studies have shown that at least in rodents, germ cell loss may not be as straight-forward as previously thought. These studies indicate that autophagy has a significant role to play in germ cell loss, particularly in the perinatal period in which it is thought to be an adaptive response to nutritional stress around birth (Rodrigues *et al*. 2009).Whether autophagy plays a role in larger mammals such as sheep and cattle, in which germ cell losses occur well before birth, needs clarification.

Two simple observations on germ cell death appear to have implications for the next developmental step, the formation of follicles. From day 55 in sheep, germ cells undergoing cell death are isolated within the ovigerous cords. While germ cells die, pre-granulosa cells that are attached to those germ cells appear to remain viable, and these 'liberated' pre-granulosa cells appear to simply become reassigned to an adjacent oocyte (Sawyer *et al*. 2002; Fig. 7C and D). In addition, before the formation of the first follicles, basal lamina was restricted to the outer walls of the ovigerous cords, and individual oocytes were separated by cytoplasmic projections of the pre-granulosa cells. The recruitment of additional pre-granulosa cells from dying germ cells results in a dramatic increase in the number of pre-granulosa cells surrounding each remaining oocyte. Simultaneously, increasing signs of basement membrane material become apparent between oocytes within the cords. Ultimately, each oocyte–pre-granulosa cell complex becomes enclosed by basement membrane and is thus separated from the cord as a new primordial follicle (Fig. 7G and H). It has been proposed that it is the increase in pre-granulosa cell numbers surrounding each

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oocyte, which provides the impetus for the formation of each follicle and for providing the cellular machinery to produce increased quantities of basement membrane material (Morrison & Marcinkiewicz 2002, Kezele & Skinner 2003, Britt *et al*. 2004). There is significant variability in size and granulosa cell number among newly formed follicles. Sheep's type 1 follicles (containing one layer of flattened granulosa cells) have been reported to have between one and eleven granulosa cells in the largest cross-sectional area examined (Lundy *et al*. 1999; Fig. 7G and H). Such variability seems likely to relate to proximity of oocytes to apoptotic germ cells during development, and as such the number of 'liberated' pre-granulosa cells acquired by each oocyte. Yet to be determined is whether manipulating the rate of germ cell apoptosis will affect the number of granulosa cells in those primordial follicles that assemble, and whether granulosa cell number in primordial follicles will affect the ultimate outcome for that follicle.

In rodents, follicle growth is first observed in the early post-natal period, while in sheep and cattle the first signs of follicle growth are observed pre-natally and can be observed as soon as follicles are isolated (Fig. 8A and B). Indeed, in both sheep and cattle, large antral follicles can be observed before birth. Two morphological criteria are generally accepted to indicate follicle growth; enlargement of the oocyte and the activation of granulosa cells from flattened squamous cells to cuboidal cells (Fig. 8A), although BrDU labeling has been detected in follicles with a complete layer of flattened granulosa cells (Fig. 8B). Once activated, follicles continue to grow through the proliferation of granulosa cells and concomitant enlargement of the oocyte, along with the establishment and growth of the thecal layers. Sawyer *et al*. (2002) reported that in sheep numerous follicles appear to form with at least some cuboidal granulosa cells already present, in conjunction with a large variation in both oocyte diameter and granulosa cells number, which harbors the potential for misclassification of newly formed follicles. While most studies of early follicular growth focus on oocytes and granulosa cells, the development of the thecal layer has been comparatively neglected until recently (Young & McNeilly 2010). While most believe that the thecal layer becomes established around type 3 stage follicles (two or more layers of granulosa cells), some have suggested that thecal cells may be present from the earliest stages of growth (Hirshfield 1991). Recent studies have indicated the presence of a population of thecal stems cells, which would support this contention (Honda *et al*. 2007, Hatzirodos *et al*. 2012). The molecular mechanisms involved in the initiation of follicular growth are still the topic of numerous studies.

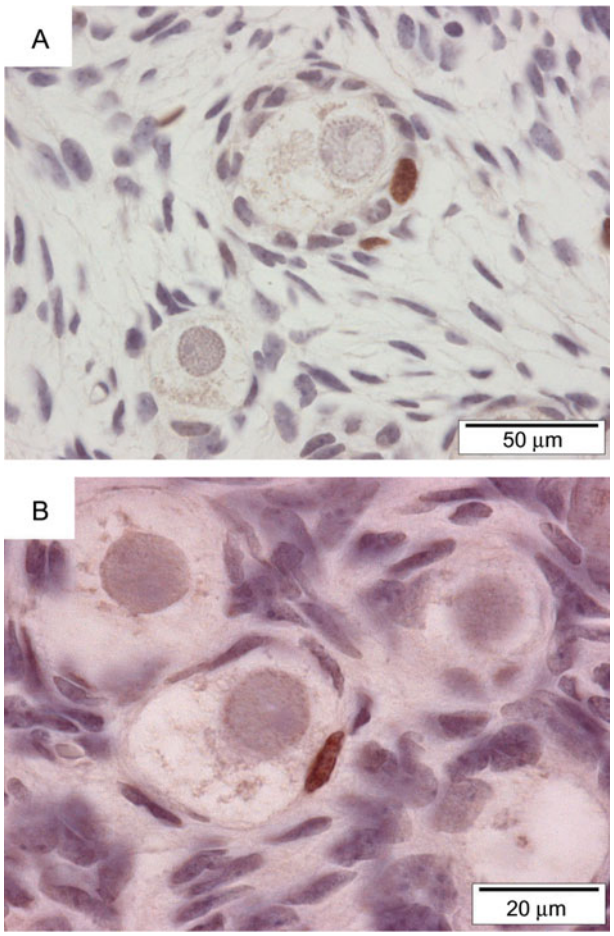

Figure 8
(A) Embryonic day 100 sheep ovary, BrdU labeling showing a follicle in the early stages of growth. Note change in the shape of granulosa cells from flattened (left hand side) to more cuboidal (right hand side). (B) Embryonic day 100 sheep ovary, BrdU labeling, showing a newly-formed follicle surrounded by flattened granulosa cells, with labeled granulosa cell indicating the first signs of follicle activation.

Post-natal implications

Since the Barker hypothesis, associating reduced fetal growth with a number of chronic post-natal conditions, was first proposed, the developmental origins of health and disease have increasingly become the focus of many studies. It is apparent that pre-natal ovarian development requires the successful completion of a number of key events, with the correct level and timing required to establish a normal functioning ovary in adult animals. Changes in the fetal environment through nutrition, exposure to contaminants, or maternal disease, have the

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potential to alter ovarian development, with major implications for fertility.

One of the most widely studied ovarian diseases is PCOS, the most common cause of anovulatory infertility in women, affecting up to 5% of women worldwide. PCOS is highly variable in its presentation and is thought to be due to both genetic and non-genetic causes. In a number of animal models, pre-natal exposure to androgens produces female offspring with symptoms largely mimicking those of PCOS (Franks 2009). The timing of the androgen exposure is important in establishing the post-natal PCOS phenotype, with exposure during the period of ovigerous cord formation, germ cell meiosis, apoptosis, and follicle formation being required to successfully produce post-natal PCOS symptoms. Throughout this period, androgen receptors are expressed in both cortical and medullary stromal tissue, notably in cell streams and rete ovarii (Juengel *et al.* 2006). Expression appears to be absent from those cells within the ovigerous cords and from small follicles. In addition, genetic linkage studies identified a region of the genome associated with PCOS (Urbanek *et al.* 2005) and a flanking gene, fibrillin 3, is expressed in ovarian stroma early during ovary development (Hatzirodos *et al.* 2011). As fibrillins regulate TGFβ activity and TGFβ stimulates fibroblast replication and collagen synthesis, it has been suggested that changes in expression of fibrillin 3 in the fetal ovary could lead to a predisposition to develop PCOS in later life (Hatzirodos *et al.* 2011). These studies also indicate that the ovarian stroma, rete, or cell streams play an important role in the establishment of PCOS. Functional differences in the thecal layer have been well characterized in PCOS (Wickenheisser *et al.* 2000). While it is well documented, but perhaps often under-appreciated, the symptoms of PCOS include a thickened tunica albuginea and cortical region (Hughesdon 1982) and these models represent an opportunity to study the roles of both the cell streams and rete ovarii. Interestingly, in a fetal-androgenized sheep model for PCOS, differences have been reported in the morphology and volume of the connecting rete from around the time when pre-natal androgen exposure is completed (Smith *et al.* 2009). This supports the earlier contention that perhaps one role of the ovarian rete is to provide cells for the ovarian cortex in late fetal life until at least early post-natal life. In another study (Smith *et al.* 2009) in sheep, using both testosterone and the non-aromatizable androgen, dihydrotestosterone, it was proposed that both androgen- and estrogen-mediated effects may play a role in the PCOS condition. A strong androgen effect was noted before puberty, resulting in increased follicle recruitment, while post puberty the effect appeared to be estrogen-mediated, manifested as follicular persistence. Again, during the androgen exposure period, both *ESR1* and *ESR2* were widely expressed in the ovary, similar to *AR*, being observed in the rete and cell streams, but unlike *AR*, strong expression was noted in the surface epithelium as well as both oocytes and pre-granulosa cells within the ovigerous cords and developing follicles. While the precise mechanism underlying the establishment of the PCOS condition is still to be determined, studies to date implicate alterations in pre-natal ovarian development and in particular highlight the importance of the relatively understudied ovarian stroma.

The original Barker hypothesis was based on the effects of gestational under-nutrition. Gestational under-nutrition has again come under the spotlight, particularly the effects on post-natal reproductive performance in domestic livestock. Studies in sheep (Rae *et al.* 2001, Lea *et al.* 2006), cattle (Martin *et al.* 2007, Mossa *et al.* 2009, Evans *et al.* 2012), and mice (Meikle & Westberg 2001) have shown that offspring from animals subjected to restricted gestational nutrition display a range of post-natal reproductive problems including lower ovulation rates, poor conception rates, lower calving to conception rates, and poor embryo survival, all factors of major significance to the livestock industry. These reproductive issues are thought to be related to ovarian function, oocyte viability, and uterine function. In addition, in cattle a similar outcome and physiology is seen in offspring born to mothers suffering from chronic infections during gestation (Evans *et al.* 2012). While there appear to be some contradictions between studies related to either species, complexity, and timing of the nutritional restriction imposed, or other technical considerations, the final outcome of reduced fertility appears to be consistent. These studies have shown that the outcome appears to be the result of perturbations in germ cell and/or follicle development and a delay in germ cell meiosis. It is not clear whether the effects are via direct effects on germ cells, or mediated by somatic cell types, or a combination of both. Evans *et al.* (2012) reports elevated maternal testosterone levels during nutritional restriction, while Murdoch *et al.* (2003) report elevated expression of the anti-apoptotic factor BCL2 around the time of germ cell death. In cattle, ovaries from animals born following a gestational nutrition challenge have low numbers of antral follicles, low plasma AMH concentrations, and a low ovarian reserve (Mossa *et al.* 2009). Similar results have been reported in rats (Bernal *et al.* 2010), while in sheep there appears to be no difference in ovarian reserve, but

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fewer growing follicles (Rae *et al*. 2001). The mechanisms underlying the development of these post-natal symptoms have been most fully studied in sheep, where it has been proposed that a delay in meiotic maturation is largely responsible (Rae *et al*. 2001, Lea *et al*. 2006). At around mid-gestation, in the nutritionally challenged fetal sheep ovary there appears to be an upregulation of the tumor suppressor/cell cycle arrest modulator p53, the base-excision repair polymerase β, the anti-apoptotic factor BCL2, and 8-oxoguanine, the benchmark for oxidative DNA modifications. Collectively, these results are indicative of oxidative damage to the fetal oogonia, brought on by maternal nutritional stress, prompt cell cycle delay and repair, and anti-apoptotic responses. The differences in ovarian reserve between studies/species may reflect the relative success of the response mechanism in being able to 'rescue' damaged germ cells. Similarly alterations in ovarian development, particularly affecting establishment of the ovarian reserve, i.e. the number of primordial follicles formed by the time of birth or during the early post-natal period, have been implicated as one pathway to establish an ovary predisposed to POF (Goswami & Conway 2005). Changes in regions of the X chromosome defined as POF loci (Therman *et al*. 1990) are thought to result in a blockage of germ cell meiosis leading to oocyte apoptosis (Burgoyne & Baker 1984). *FMR1* has high expression levels in the fetal ovary and is thought to play a role in germ cell proliferation (Bachner *et al*. 1993); mutations of this gene have been associated with POF. The absence of an X chromosome, as in Turner's syndrome, also leads to a form of POF. In these individuals, there appears to be a blockage in follicle formation resulting in widespread follicular atresia in the fetal ovary (Loughlin *et al*. 1991).

Conclusions

Studies on both PCOS and gestational under-nutrition, two common reproductive issues, illustrate how differing alterations in ovarian development (somatic cells in PCOS and germ cells in under-nutrition) affect adult fertility, and highlight the importance of ovarian development. While this review summarizes much of our understanding to date on ovarian formation it has also highlighted potential mechanisms of how environmental influences could alter the many complex processes that occur during fetal ovarian development. The challenge for this field of research is to precisely determine what and how environmental effects act on different cells and remodeling processes and when during development this can occur to alter ovarian function later in life. Achieving this may assist us in determining underlying causes of ovarian diseases.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

Funding

This review did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector, but funding of the authors is received from the National Health and Medical Research Council of Australia, the Australian Research Council, and AgResearch Core funding provided by the New Zealand Ministry for Business, Innovation, and Employment.

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Received in final form 18 March 2014
Accepted 14 April 2014
Accepted Preprint published online 16 April 2014

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-14-0062

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