

Ovarian physiology: follicle development, oocyte and hormone relationships

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Abstract

The ovarian follicle is the fundamental unit of the ovary. It contains the oocyte that may eventually ovulate, undergo fertilization and form an embryo. It also provides the steroid and protein hormones required for maintenance of the ovarian cycle, the secondary sex characteristics and preparation of the uterus for implantation. Follicle formation and folliculogenesis have been well documented for many mammalian species. However, the control of follicular reserves and entry of follicles into the growth path towards atresia or ovulation are not well understood. We have investigated several aspects of follicle formation and folliculogenesis by (a) using unbiased assumption-free stereological methods to accurately count follicles, particularly primordial follicles in the follicular reserve, (b) testing the effects of members of the transforming growth factor- β family on folliculogenesis and follicle function, and (c) examining the role of estrogen in folliculogenesis using the aromatase knock-out mouse model. These studies are summarized and reviewed.

Keywords: estradiol, folliculogenesis, mice, TGF- β .

Introduction

The ovarian follicle is the fundamental unit of the ovary. It contains the oocyte that may eventually ovulate, undergo fertilization and form an embryo. It also provides the steroid and protein hormones required for maintenance of the ovarian cycle, the secondary sex characteristics and preparation of the uterus for implantation, and after ovulation, the corpus luteum provides the hormones essential for establishment and maintenance of pregnancy. Follicle formation and folliculogenesis have been well documented for many mammalian species. However, the control of follicular reserves (Tilly and Rueda, 2008) and entry of follicles into the growth path towards atresia or ovulation (Findlay *et al.*, 2002) are not well understood. We have investigated several aspects of follicle formation and folliculogenesis by (a) using unbiased assumption-free stereological methods to accurately count follicles, particularly primordial follicles (Kerr *et al.*, 2006), (b) testing the effects of members of the transforming growth factor- β superfamily on folliculogenesis and follicle function (Rosairo *et al.*, 2008), and (c) examining the role of estrogen in folliculogenesis using the aromatase knock-out mouse model (Britt *et al.*, 2000, 2004a, b; Liew *et al.*, 2008).

The primordial follicle pool

Follicles form in the ovary when developmentally arrested germ cells or oocytes in nests individually acquire somatic follicular cells and organize into discrete 'resting' follicles. This event occurs in late pregnancy or early in the postnatal period in most mammals. The oocytes in the primordial follicles formed in this way remain in the diplotene stage of the first meiotic prophase until they enter the growth phase, up to 40-50 years later in women. It is a widely held view that the mammalian neonatal ovary contains a finite stockpile of non-growing primordial follicles (Zuckerman, 1951). Commencing at or soon after birth, a small number of these primordial follicles enter the growth path during which they either degenerate (mainly by atresia) or complete maturation and ovulate (<1%). As a result the supply of follicles is said to decline with age until the pool is exhausted at advanced age. The notion of a fixed, non-renewable reserve of primordial follicles in the mammalian ovary has been questioned recently, particularly with the proposal that intra and extraovarian germline stem cells could replenish oocytes and form new primordial follicles (Johnson *et al.*, 2004, 2005). If this is correct, we hypothesized that total primordial follicle numbers should remain relatively constant, at least for a significant part of adult reproductive life.

We quantified all healthy follicles in C57BL/6 mouse ovaries from postnatal day 1 to 200 using unbiased stereological methods, immunolabelling of oocyte meiosis (germ cell nuclear antigen, GCNA) and ovarian cell proliferation (proliferating cell nuclear antigen, PCNA) and electron microscopy (Kerr *et al.*, 2006). Day 1 ovaries contained 7924 ± 1564 (S.E.M.) oocytes or primordial follicles, declining on day 7 to 1987 ± 203 , with 200–800 oocytes ejected from individual ovaries into the ovarian bursa on that day and day 12. Discarded oocytes and those subjacent to the surface epithelium were GCNA-positive indicating their incomplete meiotic maturation. From day 7 to 100, mean numbers of primordial follicles per ovary were not significantly different by ANOVA, but declined by 200 days to about 10% of those levels. Primordial follicle oocytes were PCNA-negative (Kerr *et al.*, 2006).

It could be argued that our data support the hypothesis of postnatal follicle renewal in postnatal and adult ovaries of C57BL/6 mice, at least between days 7 and 100. However, several possibilities remain. We and others have found no evidence for ovarian germline

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stem cells in the ovary (Bristol-Gould *et al.*, 2006; Eggan *et al.*, 2006; Kerr *et al.*, 2006). Secondly, the counting method, despite being the 'gold standard', may not have been accurate enough (i.e., there were insufficient numbers of ovaries counted to reduce the error), and the method of statistical analysis may not have been robust enough to detect a small but significant decline in primordial follicle numbers during this period, as argued by Faddy and Gosden (2007). However, we have confirmed the relatively stable number of primordial follicles during this period in adult C57Bl6 mice in a recent study, and analysis of this and our earlier data (Kerr *et al.*, 2006) using regression analysis did not reveal a significant decline in numbers of primordial follicles (Kerr *et al.*, unpublished data). This leads to the third possibility that there may be very low, and so far undetectable, rates of follicle exit from the pool up to day 100, and thereafter the rate increases for reasons that are not clear.

Further research is needed to examine the factors regulating the size of the primordial pool during reproductive age. The Tilly laboratory claimed that primordial follicle numbers in ovaries of adult C57Bl6 mice treated with chemical toxins that destroy the oocytes of primordial follicles, recovered primordial follicle numbers to pretreatment levels within 24-36 hr (Johnson *et al.*, 2005). They concluded that this is evidence for follicle renewal. We have repeated these studies using doxorubicin or trichostatin A and a more rigorous method of counting follicles (Kerr *et al.*, 2006), and were unable to demonstrate either by immunohistochemical staining or stereological analysis that regeneration of primordial follicles occurred after drug treatment (Kerr *et al.*, unpublished observations), arguing against oocyte renewal. A recent study using ovarian transplants in mice, with or without radiation to destroy primordial follicles, found no evidence to support the hypothesis that progenitor cells from extra-ovarian sources can repopulate the adult ovary (Begum *et al.*, 2008).

We also need to understand the reasons for the rapid decline in follicle numbers after day 100, an event similar to that in women approaching the menopause. Of interest in this regard is a recent study by Livera *et al.* (2008) showing that a p63 null mutation protects mouse oocytes from radio-induced apoptosis. The transactivation isoform of p63 (Tap63 α), not p53, is expressed in female germ cells and is essential in a process of DNA-damage-induced oocyte death (Suh *et al.*, 2006). This raises the interesting question about the degree of DNA damage of primordial oocytes and the effect this may have on fertilizing capacity of older oocytes and their maintenance in the ovary.

We conclude that there is mounting evidence against follicle renewal after birth in the mouse, although we do not have an explanation for the relatively stable numbers of primordial follicles we observed between days 7 and 100. Either way, the

proposal that regeneration does occur has generated a renewed interest in regulation of the size of the primordial pool which varies enormously between strains and within strains over time for reasons that remain unclear. The role of p63 in oocyte quality and quantity deserves further investigation.

The role of TGF- β superfamily members in folliculogenesis

Local as well as peripheral hormones or growth factors are known to influence folliculogenesis, although the identity and action of many of the local factors are not known (Findlay *et al.*, 2002). They include members of the TGF- β superfamily which includes TGF- β s, inhibins, activins, bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs) (Knight and Glister, 2006). Although TGF- β ligands and receptors have been reported in the ovaries of a range of species (Knight and Glister, 2006; Rosairo *et al.* 2008), the direct effects of TGF- β ligands on follicular development have not received much attention. The effects of null mutations in the TGF- β ligand genes on fertility have been difficult to address because the animals either die during gestation or at weaning (TGF- β 1) or exhibit perinatal lethality (TGF- β 2 & 3; Dunker and Kriegstein, 2000). Ingman *et al.* (2006) reported that the TGF- β 1 null mutants bred on a *Scid* background to reduce the inflammatory response, had severely impaired fertility due to irregular ovulation, and a reduction in oocyte number and developmental competence. Liu *et al.* (1999) recorded age-specific effects of TGF- β 1 on follicles *in vitro* with only the diameters of preantral follicles from adult mice increasing in size. We investigated the potential for TGF- β 1 to influence ovarian follicular growth and differentiation using postnatal and immature ovarian models (Rosairo *et al.*, 2008). TGF- β 1 ligand and receptor mRNAs were present in the rat ovary 4-12 days after birth and at day 25. In order to assess the impact of TGF- β 1 on follicle growth and transition from the primordial through to the primary and preantral stages of development, we established organ cultures with 4 day old rat ovaries, similar to the method of Nilsson *et al.* (2001). After 10 days in culture with FSH, TGF- β 1, or a combination of the two, ovarian follicle numbers were counted and an assessment of atresia was undertaken using TUNEL. Preantral follicle numbers declined significantly when treated with the combination of FSH and TGF- β 1, consistent with our morphological appraisal suggesting an increase in atretic primary and preantral follicles. To investigate the mechanisms behind the actions of TGF- β 1 we isolated granulosa cells and treated them with FSH and TGF- β 1. Markers of proliferative, steroidogenic and apoptotic capacity were measured by real-time PCR. Cyclin D2 mRNA expression by granulosa cells was significantly increased in response



to the combination of FSH and TGF- β 1. The expression of FKHR mRNA by granulosa cells was significantly reduced in the presence of both FSH and TGF- β 1, individually and in combination regimes. In contrast, the expression of steroidogenic enzymes/proteins was largely unaffected by TGF- β 1. These data suggest an inhibitory role for TGF- β 1 (in the presence of FSH) in preantral follicle development and progression, which most likely involves an increase in apoptosis at the primary and preantral stages of follicle development (Rosairo *et al.*, 2008).

We are now planning to examine the effects of other members of the TGF- β superfamily beginning with activin A *in vitro*. We also plan to examine the effects of manipulating levels of activin *in vivo* using follistatin knock in models (Lin *et al.*, 2008) on the ovarian phenotype of newborn and adult animals.

Role of estrogen in folliculogenesis

Gonadotrophins and steroid hormones are vital in controlling the cyclical pattern of ovarian follicular development, essential for fertility. Estrogens in particular are synonymous with fertility and infertility in mammals. Mouse models with targeted disruption of the genes encoding the estrogen receptors (ER α , ER β or both) or aromatase, the enzyme responsible for estrogen synthesis, have thrown new light on the action of estrogens in mammalian reproduction. In particular, these models provide for the first time, animals which can either respond to endogenous or exogenous estrogen (ER knockouts), or respond to exogenous estrogen but cannot make estrogen (aromatase knockout or ArKO). Although not obligatory for survival after birth or formation of the reproductive tract, estrogen was shown to be essential for both male and female fertility.

ArKO female mice are infertile. They have a block in folliculogenesis at the early antral stage and an absence of corpora lutea because of anovulation. Antral follicles are atretic and often appear as hemorrhagic cysts and the somatic granulosa and theca cells of the ovary transform into Sertoli and Leydig-like cells, respectively. The uteri are undeveloped due to the lack of estrogen. ArKO females also have elevated levels of circulating gonadotrophins and testosterone (Fisher *et al.*, 1998; Britt *et al.*, 2002, 2004a, b). Estrogen replacement in ArKO mice partially restored normal folliculogenesis and ovarian gene expression, suppressed serum gonadotrophins to within the normal range and increased the uterine weights (Britt *et al.*, 2004a, b). The possibility remains however, that some or all of the ovarian phenotype in the ArKO mouse could be due to the actions of elevated gonadotrophins or inappropriate actions of androgens on the ovary.

Therefore, we recently conducted a study to compare the effects of E2 (estradiol-17 β) replacement, or acyline (GnRH antagonist to lower gonadotrophins) or flutamide (anti-androgen) treatment on reproductive

parameters of ArKO female mice (Liew *et al.*, 2008). WT and ArKO female mice (C57B6/J129; 16 weeks old; n = 6-8/group) were assigned as follows: group 1 received either E2 (0.05 mg) pellet or placebo, group 2 received either a single s.c. injection of acyline (1.5 mg/kg/week) or placebo, and group 3 received either flutamide (25 mg pellet) or placebo for 3 weeks. Mice were subjected to daily vaginal smears. The ovaries and uterine horns were collected and weighed. One ovary and the uterine horns were fixed in formalin for histological assessment, while the other ovary was snap frozen in Ultraspec solution for RNA isolation and gene expression studies. Serum was collected for hormone measurements. The analyses are still underway. The results to date suggest that E2 replacement, but not acyline or flutamide, reversed the abnormal reproductive phenotype of the ArKO female mice. This is consistent with the reproductive phenotype of the ArKO female mouse resulting from a lack of estrogen and not the elevated circulating levels of gonadotrophins and testosterone.

We conclude that estrogen is essential for normal folliculogenesis beyond the antral stage and for maintenance of the female phenotype in ovarian somatic cells. This challenges the paradigm that the ovary is the default gonad due to the absence of 'testicular' signals, and shows the plasticity of the adult female gonad. We are now exploring the mechanisms by which estrogen exerts these actions by identifying estrogen-dependent genes in the ovary.

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