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Consequences of RAS and MAPK activation in the ovary: the good, the bad and the ugly

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Abstract

This review summarizes studies providing evidence 1) that endogenous RAS activation regulates important physiological events during ovulation and luteinization 2) that expression of the mutant, active KRAS^{G12D} in granulosa cells *in vivo* causes abnormal follicle growth arrest leading to premature ovarian failure and 3) that KRAS^{G12D} expression in ovarian surface epithelial (OSE) cells renders them susceptible to the pathological outcome of transformation and tumor formation. These diverse effects of RAS highlight how critical its activation is linked to cell- and stage-specific events in the ovary that control normal processes and that can also lead to altered granulosa cell and OSE cell fates.

Keywords

RAS; MAPK3/1; MAPK14; PI3K; C/EBP; granulosa cells; ovulation; ovarian cancer

Introduction

The major functions of the mammalian ovary are to ensure the timely release of mature oocytes and the maintenance of fertilized, implanted embryos. These functions are controlled by intraovarian and extraovarian regulatory factors that stimulate follicular growth, ovulation and luteinization (Eppig, 1991, Richards, 2001). Critical intraovarian factors are produced within each growing follicle by the oocyte and its surrounding somatic cells, the cumulus cells, that comprise a unique microenvironment (Matzuk *et al.*, 2002). Critical extraovarian factors are the pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Hunzicker-Dunn *et al.*, 2006, Richards, 1994). These hormones bind their cognate G-protein coupled receptors FSHR and LHCGR, respectively, and thereby activate adenylyl cyclase, increase intracellular cAMP and stimulate protein kinase A (PKA) activation (Hunzicker-Dunn *et al.*, 2006). Although the canonical cAMP and PKA pathway is critical for many ovarian cell functions, recent studies indicate that the signaling events controlled by FSH and LH in ovarian granulosa cells involve many additional signaling cascades, including SRC tyrosine kinase, RAS, protein kinase B (PKB/AKT) and mitogen-activated protein kinases (MAPKs) (Fan *et al.*, 2010, Gonzalez-Robayna

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et al., 2000, Hunzicker-Dunn *et al.*, 2006, Richards, 2001, Wayne *et al.*, 2007). In this review, we will focus primarily on the roles of LH, RAS and MAPK signaling cascades in preovulatory follicles that are essential for ovulation and luteinization; that is, the good physiological consequences of RAS (Figures 1 and 2). We will also discuss how inappropriate activation of RAS can lead to deleterious (bad) effects in granulosa cells as well as the oncogenic (ugly) transformation of ovarian surface epithelial (OSE) cells (Figures 1 and 2).

The physiological consequences of RAS activation in the ovary

LH stimulation of ovulation has been recognized for many years and shown to be dependent on the induction of LH receptors in granulosa cells (Richards, 1979, Richards, 1994). Furthermore, many mutant mouse models have shown convincingly that successful ovulation also depends on expansion of the cumulus cell-oocyte complex (Richards, 2006), a process that requires the synthesis and stabilization of a hyaluronan rich extracellular matrix (Richards, 2006, Richards *et al.*, 2010). Because cumulus cells express few if any receptors for LH and fail to respond when directly stimulated by LH in culture, the mechanisms by which the LH surge induces COC expansion and oocyte maturation have remained enigmatic. However, the recent pioneering studies of Espey, Conti, Shimada, Fan, Liu and Nautiyal (Conti *et al.*, 2005, Espey *et al.*, 2002, Fan *et al.*, 2009, Hernandez-Gonzalez *et al.*, 2006, Hsieh *et al.*, 2007, Liu *et al.*, 2010, Nautiyal *et al.*, 2010, Park J *et al.*, 2004, Shimada *et al.*, 2005, Shimada *et al.*, 2006) have unveiled the critical roles of the EGF-like factors amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) in mediating LH actions in preovulatory follicles. Specifically, the EGF-like factors are induced rapidly by LH (or its analog human chorionic gonadotropin (hCG)) in granulosa cells *in vivo* and in culture. AREG and EREG are potently up-regulated in granulosa cells by PKA and by mechanisms that require the transcriptional regulatory factor NRIP1 (also known as RIP140) (Nautiyal *et al.*, 2010).

In addition, the induction of the EGF-like factors in granulosa cells and cumulus cells is differentially regulated by MAPK14 (also known as p38MAPK) (Liu *et al.*, 2010). Inhibitors of MAPK14 can block COC expansion and the expression of specific genes including *Ptgs2* that controls the synthesis of prostaglandin E2 (PGE2). The physiological relevance of the inhibitor studies was confirmed using mice in which the *Mapk14* gene was disrupted selectively in granulosa cells and cumulus cells (LiuZ *et al.*, 2010). Specifically, COC expansion failed to occur in response to either FSH or PGE2 in the *Mapk14* null COCs and the expression of many genes involved in this process, including *Areg* and *Ereg*, was impaired. However, when the *Mapk14* null COCs were cultured with AREG, expansion was normal indicating that the critical function of MAPK14 in COCs is to regulate the PGE2 induction of *Areg* and *Ereg*. Conversely, MAPK14 was not essential for the expression of the *Areg* and *Ereg* genes in granulosa cells. Rather, *Mapk14* null granulosa cells expressed elevated levels of *Areg* and *Ereg*, presumably because PKA was the major kinase regulating the induction of these genes in granulosa cells. Because the *Mapk14* mutant mice are fertile, AREG and EREG produced by the granulosa cells appear sufficient to by-pass and compensate for the markedly reduced expression of these genes in the cumulus cells to permit COC expansion to occur in a normal manner (Liu *et al.*, 2010).

Once induced, the EGF-like factors are thought to function by both autocrine and paracrine mechanisms to propagate the LH signal to granulosa cells and cumulus cells to induce ovulation, cumulus cell-oocyte complex (COC) expansion, oocyte maturation and luteinization (Hsieh *et al.*, 2007, Park *et al.*, 2004, Shimada *et al.*, 2006). The growth factor effects are mediated by the EGF receptor (EGFR, also called ERBB1), because EGFR tyrosine kinase inhibitors and disruption of the EGF signaling network *in vivo* and in culture

block oocyte maturation, COC expansion and ovulation (Hsieh *et al.*, 2007, Reizel *et al.*, 2010, Shimada *et al.*, 2006). In cumulus cells expression and activation of the EGF receptor is dependent on the oocyte and oocyte-derived factors BMP15 and GDF9 (Su *et al.*, 2010). Moreover, prolonged activation (greater than 2h) of the EGF receptor is essential for these successful completion of these events (Reizel *et al.*, 2010). In this regard, recent studies show that an additional member of the EGF-like factor family, neuregulin 1 (NRG1) Type III transcript is induced in granulosa cells of preovulatory follicles and acts coordinately with AREG/EREG to enhance granulosa cell differentiation and oocyte maturation and development (Noma *et al.*, 2010). Recent studies also indicate that reactive oxygen species generated during the inflammatory response that occurs in the follicle in response to the LH surge may also activate the RAS/MAPK pathway and thereby play a critical role in ovulation (Shkolnik *et al.*, 2011). Activation of the RAS/MAPK pathway also plays a key role in oocyte maturation by regulating the production of cGMP and its transport to the oocyte where it serves as a potent inhibitor of phosphodiesterase 3A (Vaccari *et al.*, 2009, Zhang *et al.*, 2010).

Of physiological relevance, activation of RAS in granulosa cells by the gonadotropins has also been documented *in vivo* (Fan *et al.*, 2008, Fan *et al.*, 2009). The levels of active, GTP-bound RAS are undetectable in ovaries of immature mice, increase slightly in response to the FSH-like gonadotropin eCG (equine chorionic gonadotropin) that stimulates preovulatory follicle development but are increased dramatically by 2–4 h after hCG treatment that stimulates ovulation. This resembles a “hit and run” reaction since hCG also induces significant phosphorylation of the EGF receptor, MEK1/2, and ERK1/2, all maximal at 2–4 hours post-hCG (Fan *et al.*, 2008, Fan *et al.*, 2009). Once the RAS pathway is activated, granulosa cells are irreversibly committed to ovulation and terminal differentiation to luteal cells. Specifically, the pioneering studies of Cornelia Channing (Channing, 1970) showed that if granulosa cells of preovulatory follicles were exposed to LH *in vivo* they would undergo spontaneous luteinization when placed in culture. Likewise, rat granulosa cells will luteinize in culture if exposed *in vivo* to LH/hCG for 5–7h (Oonk *et al.*, 1989).

The functions of LH and RAS are mediated, in part, by the downstream kinases MAPK3/1 (also known as ERK1/2), because pharmacological inactivation these enzymes or disruption of the genes encoding these kinases in granulosa cells causes dramatic changes in granulosa cell fate (Fan *et al.*, 2009). Although follicular development is normal in the *Erk1/2* mutant mice, they are infertile because ovulation, COC expansion, oocyte maturation as well as luteinization are blocked (Fan *et al.*, 2009). The effects of disrupting *Erk1/2* in granulosa cells demonstrate the critical importance of ERK1/2 activation in mediating LH action at the ovulatory stage of follicular development *in vivo* (Fan *et al.*, 2009, Hsieh *et al.*, 2007, Panigone *et al.*, 2008). Additionally, the transcription factor CAAT/Enhancer Binding Protein beta (C/EBP) is a target of ERK1/2 and appears to control some of the cell fate decisions in granulosa cells of ovulatory follicles (Fan *et al.*, 2009, Fan *et al.*, 2011, Sterneck *et al.*, 1997). In particular, data obtained from mutant mice in which the genes encoding CCAAT/enhancing binding proteins alpha and beta (*Cebpa* and *Cebpb*) have been disrupted selectively in granulosa cells show that these two transcription factors regulate specific genes that control the neovascularization of the follicle during the process of luteinization (Fan *et al.*, 2011). LRH1 (nuclear receptor liver receptor homolog 1 also known as NR5A2) is a transcription factor highly similar in structure to Steroidogenic Factor 1 (SF1; NR5A1). Although both are expressed in granulosa cells of growing follicles, recent studies indicate that LRH1, rather than SF1, is a potential RAS-ERK1/2 target in granulosa cells of preovulatory follicles. When the *Lhr1* gene is conditionally disrupted in granulosa cells, the mutant mice show ovulation and luteinization defects (Duggavathi *et al.*, 2008) whereas conditional disruption of *Nr5a1* in granulosa cells selectively impairs early stages of follicle

growth (Jeyasuria *et al.*, 2004). In addition, LHR1 is known to be phosphorylated and functionally activated by ERK1/2 (Lee *et al.*, 2006).

These observations provide strong evidence that RAS activation is an important physiological mediator of gonadotropin signaling in ovarian follicles, especially at the time of ovulation when activation of ERK1/2 is required not only for follicle rupture but also for oocyte meiotic maturation, COC expansion and luteinization (Fan *et al.*, 2009, Panigone *et al.*, 2008). Thus, at the preovulatory stage of follicular growth, RAS and ERK1/2 activation direct the terminal differentiation of granulosa cells to non-dividing luteal cells (Espey *et al.*, 2002, Robker *et al.*, 1998, Robker *et al.*, 1998)(Figures 1 and 2). Because follicular growth was normal in mice in which ERK1/2 were depleted in granulosa cells, one might predict that either RAS or ERK1/2 are not activated at early stages of follicular growth or are not essential. Studies in culture indicate that RAS, ERK1/2 and MAPK14 can be activated by FSH and AREG in granulosa cells isolated from follicles at earlier stages of growth (Wayne *et al.*, 2007). Importantly, RAS-GTP levels are transiently increased after FSH or AREG stimulation whereas a dominant negative RAS mutant blocked both MAPK (including ERK1/2 and p38MAPK) phosphorylation and the differentiation of granulosa cells to luteal-like cells induced by FSH or AREG in culture (Wayne *et al.*, 2007). However, these studies in culture did not address the effects of activating RAS in granulosa cells at early stages of follicle growth *in vivo*.

Granulosa cell-specific *Kras*^{G12D} mutation causes stage-dependent defects in granulosa cell fate

To specifically activate RAS protein in granulosa cells of small growing follicles *in vivo*, we generated conditional knock-in mouse models, using the *Loxp-Stop-Loxp Kras*^{G12D} mouse strain (Tuveson *et al.*, 2004) and selected Cre strains in which the granulosa cells express constitutively active *Kras*^{G12D} at specific stages of follicular growth (Fan *et al.*, 2008). *Amhr2-Cre* is expressed in granulosa cells of small and growing follicles as well as ovarian surface epithelial (OSE) cells (Fan *et al.*, 2009, Hernandez *et al.*, 2009, Jorge *et al.*, 2004). By contrast, *Cyp19-Cre* is expressed in a subset of granulosa cells in small follicles and is highest in granulosa cells of antral and preovulatory follicles but is absent from OSE cells (Fan *et al.*, 2008, Fan *et al.*, 2008). Because the progesterone receptor (*Pgr*) is only expressed in granulosa cells of preovulatory follicles exposed to the LH surge, Cre recombinase is only active in granulosa cells following the LH surge in the *Pgr-Cre* mice (Soyal *et al.*, 2005).

The *Kras*^{G12D};*Amhr2-Cre* and *Kras*^{G12D};*Cyp19-Cre* conditional mutant mice become infertile by 3 months of age (Fan *et al.*, 2008) and exhibit symptoms similar to premature ovarian failure (POF) in human patients. The ovaries of the *Kras*^{G12D};*Amhr2-Cre* and *Kras*^{G12D};*Cyp19-Cre* mice contain numerous small abnormal follicle-like structures in which the granulosa cells are non-mitotic, non-apoptotic and fail to express known markers of granulosa cell differentiation, including FSH and LH receptors (Fan *et al.*, 2008) (Figures 1 and 2). As a consequence of KRAS^{G12D} expression, their development is arrested at a very early stage of follicle growth and they fail to either differentiate or die. The formation of these abnormal follicle-like structures was dependent on the stage of granulosa cell differentiation and follicle development because the abnormal follicles were not observed in the *Kras*^{G12D};*Pgr-Cre* mutant mice where recombination only occurs after the LH surge. Moreover, the absence of any ovarian defects in the *Kras*^{G12D};*Pgr-Cre* mutant mice indicates that constitutively active KRAS does not impact the fate of granulosa cells that have already been exposed to RAS activation initiated by the LH surge.

Although the detailed mechanisms by which endogenous activation of *Kras*^{G12D} in *Kras*^{G12D};*Amhr2*-Cre and *Kras*^{G12D};*Cyp19*-Cre mice lead to cell cycle arrest of granulosa cells remain unknown, recent results indicate that in granulosa cells of growing follicles this may involve rapid silencing of *Kras* gene itself and the up-regulation of tumor repressor *Pten* (Fan *et al.*, 2009) (Figure 1). Loss of KRAS and increased PTEN lead secondarily to the absence of both phospho-ERK1/2 and phospho-AKT in the mutant granulosa cells (Fan *et al.*, 2009). Because activation of the endogenous *Kras*^{G12D} mutant gene in cultured granulosa cells by Cre-mediated DNA recombination did not induce cell cycle arrest, the stage of follicular development and granulosa cells differentiation dictated by the follicular microenvironment appear to be crucial for the *Kras*-induced inhibitory effects on granulosa cell proliferation (Fan *et al.*, 2009). Furthermore, it is important to note that the apparent silencing of the endogenous *Kras*^{G12D} gene and cell cycle arrest in granulosa cells of the *Kras*^{G12D};*Amhr2*-Cre mice *in vivo* is specific for granulosa cells and does not occur in the ovarian surface epithelial (OSE) cells present in the ovaries of the same *Kras*^{G12D};*Amhr2*-Cre mice (Figures 1 and 2). As will be discussed below, the response of OSE cells to *Kras*^{G12D} is distinctly different from that of granulosa cells (Figures 1 and 2).

Pathological consequences of *Ras* mutations lead to ovarian surface epithelial cancer

Because the tumor suppressor PTEN was expressed at elevated levels the abnormal follicles that contained non-dividing and non-differentiated granulosa cells of the *Kras*^{G12D};*Amhr2*-Cre and *Kras*^{G12D};*Cyp19*-Cre mice, we sought to determine if we disrupted the *Pten* gene we might be able to restore normal follicular development. Strikingly, the *Pten*^{fl/fl};*Kras*^{G12D} double mutant mice do not develop granulosa cell tumors (GCTs). Rather, the abnormal follicle-like structures containing granulosa cells in cell cycle arrest form as in the *Kras* single mutant mice, and are not rendered tumorigenic by disruption of the *Pten* gene (Fan *et al.*, 2009). That the *Kras*^{G12D}/*Pten* mutant granulosa cells do not undergo transformation indicates that these cells are extremely resistant to this specific combination of oncogenic insults. These results confirm those of others indicating that granulosa cells are highly resistant to certain oncogenes (Connolly *et al.*, 2003, Orsulic *et al.*, 2002). Thus, these cells appear to have robust self-protecting mechanisms against these specific mutations to limit cell cycle progression, but at the expense of normal reproductive functions.

However, the *Pten*^{fl/fl};*Kras*^{G12D};*Amhr2*-Cre double mutant mice develop ovarian surface epithelial (OSE) cell derived tumors (Fan *et al.*, 2009). The specificity of OSE tumor formation is due to the expression of the *Amhr2* but not *Cyp19* in OSE cells. Based on histological criteria, the tumors in the *Pten*^{fl/fl};*Kras*^{G12D};*Amhr2*-Cre mutant mice are classified as low-grade ovarian serous papillary adenocarcinomas (Mullany *et al.*, 2011). The tumors appear at an early age and with 100% penetrance. Thus, this is the first mouse model in which to study this ovarian cancer subtype. The mutant OSE cells express specific müllerian epithelial marker genes, grow in soft agar and develop ectopic invasive tumors in recipient mice, indicating that the cells are transformed. Two key hallmarks of the mutant OSE cells are the elevated expression of the tumor suppressors *Trp53* (*p53*) and its microRNA target, *miR-34a-c* indicating that elevated TRP53 and miR-34a-c may exert negatively regulatory effects that reduce the proliferative potential of OSE cells leading to the low-grade serous adenocarcinoma phenotype (Mullany *et al.*, 2011).

OSE cancers make up nearly 90% of all ovarian cancers. In contrast, only 5% of human ovarian cancers are derived from granulosa cells (Cho *et al.*, 2009, Vanderhyden, 2005). This seems unexpected because granulosa cells are the most abundant proliferative cell type in growing follicles. The low incidence of granulosa cell tumors (GCTs) is likely due to the strong resistance of granulosa cells to specific oncogenic stimuli. In addition, granulosa cells

are removed routinely and systematically from the growing pool by apoptosis or by terminal differentiation to non-dividing luteal cells (luteinization). Thus, unlike many other cell types, granulosa cells may escape most of the rare occurrences of oncogenic mutations. One recent exception appears to be the transcription factor *Foxl2* that is important for early follicle development and granulosa cell proliferation. A specific mutation that occurs with high frequency is observed in a high percentage of human adult GCTs (Kobel *et al.*, 2009, Shah *et al.*, 2009). Known mutant mouse models exhibiting granulosa tumor development include inhibin null mice (Li *et al.*, 2007, Matzuk *et al.*, 1992), granulosa cell-specific *Smad1/5/8* knockout mice (Pangas *et al.*, 2008), and granulosa cell-specific β catenin mutant mice (Boerboom *et al.*, 2005, Boerboom *et al.*, 2006, Boyer *et al.*, 2009, Boyer *et al.*, 2009, Lague *et al.*, 2008, Richards *et al.*, 2011). To what extent any of these pathways are altered in the *FOXL2* mutant human GCTs remains to be determined.

Clinical Implications

Based on the foregoing review, it is clear that the role of RAS activation in the ovary is cell and context specific. The activation of RAS is critical for initiating LH-mediated events, such as ovulation and luteinization, and in this context RAS activated MAPK3/1 act as a critical molecular switch that reprograms granulosa cell functions. The FSH driven molecular program that sustains follicular growth is turned off and the terminal differentiation of granulosa cells to non-dividing luteal cells is turn on. Inappropriate expression of a mutant constitutively active form of KRAS (KRAS^{G12D}) leads to granulosa cell cycle arrest and impaired follicle growth. Thus, mutations in RAS could be associated with some unknown causes of premature ovarian failure POF, contribute to polycystic ovarian syndrome (PCOS), as well as render OSE cells susceptible to transformation, leading to ovarian cancer. Because POF is often associated with elevated levels of FSH and because FSH can activate RAS in granulosa cells in culture and *in vivo* (albeit to a lesser extent than LH), it is possible that prolonged stimulation of granulosa cells by FSH could inappropriately activate RAS, thereby impairing granulosa cell functions leading to cell cycle arrest. Importantly, prolonged exposure of mouse ovaries to FSH does not lead to ovarian cancer (Kharbanda *et al.*, 1998). In PCOS, the levels of LH may activate RAS inappropriately in theca and granulosa cells altering their developmental process. Chronic levels of LH have been shown to cause benign ovarian luteomas in mice (Risma *et al.*, 1997) but have not been reported in women. Curiously, the RAS mutations that impact granulosa cells and OSE cells do not cause testicular tumors but other aspects of male fertility have not been examined in detail. In summary, the most important physiological function of RAS in the gonads is its activation in the ovary at the time of ovulation. In this regard, it is important to note that activation of ERK1/2 by GnRH is essential for stimulating the LH surge. Although not yet known, activation of ERK1/2 in pituitary cells by elevated GnRH is likely to be a RAS dependent event as well (Bliss *et al.*, 2009). Although female mice deficient for *Mapk3/1* in gonadotropes are infertile and fail to exhibit LH surges, ERK1/2 is not critical for basal LH secretion in male mice that remain fertile. The most important pathological effects of RAS activation in the ovary appear to be the potent ability of mutant RAS to cause cell cycle arrest in granulosa cells and to facilitate OSE cancer.

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Figure 1.

Panel A LH binding to the LH receptor (LHR) leads to induction of the EGF-like factors (AREG/EREG) and activation of the RAS/ERK1/2 pathway in granulosa cells of preovulatory follicles (blue letters). This cascade is essential for events associated with ovulation and luteinization in which proliferating granulosa cells terminally differentiate into non-dividing luteal cells (also see Richards *et al*, 2010). **Panel B:** Premature expression of activated KRAS^{G12D} in granulosa cells of small growing follicles of the *Kras^{G12D}* mutant mice is presumed to stimulate PI3K and phosphorylation of AKT as well as the phosphorylation and activation of ERK1/2 (black arrows). However, by mechanisms that are not yet known, the expression of KRAS^{G12D} leads to rapid silencing the endogenous *Kras*/*Kras^{G12D}* allele (red line). This in turn prevents: 1) ERK1/2 phosphorylation, 2) activation of PI3K and 3) phosphorylation of AKT (red arrows). Activation of PI3K/AKT is also reduced by the dramatic up-regulation of PTEN that occurs in the KRAS^{G12D} mutant cells (red lines). Depletion of *Pten* in the *Kras^{G12D}* mutant granulosa cells does not alter the growth-arrested phenotype of the granulosa cells. **Panel C:** By contrast, expression of KRAS^{G12D} or disruption of *Pten* alone has little or not effects on OSE cells. However, the loss of *Pten* in the *Kras^{G12D}* mutant mice causes hyper-activation of the ERK1/2 and PI3K/AKT pathways leading to OSE cell transformation and the rapid growth of serous adenocarcinomas (green arrows) (Fan *et al*, 2010; Fan *et al*, 2009).

Figure 2.

Cell context specific effects of RAS activation in the ovary. **A.** Histological image of a normal mouse ovary showing a preovulatory (PO) follicle that is capable of ovulating in response to an LH surge. The LH surge activates the RAS, MEK1, MAPK3/1 (ERK1/2) pathway that controls ovulation, COC expansion, oocyte maturation and luteinization. These are the good functions of RAS. **B.** Premature exposure of granulosa cells of small follicles to mutant active KRAS^{G12D} leads to the formation of abnormal follicles devoid of oocytes. The granulosa cells within these follicles do not proliferate, differentiate or undergo apoptosis. These are the bad functions of RAS. **C.** Depletion of *Pten* in ovarian surface epithelial (OSE) cells expressing KRAS^{G12D} driven by *Amhr2Cre* leads to OSE cell transformation and serous adenocarcinomas. These are the ugly outcomes of activating RAS. Similar mutations in granulosa cells within the same ovary do not lead to granulosa cell tumors (Fan *et al*, 2009).