Minireview: Physiological and Pathological Actions of RAS in the Ovary

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The small G proteins of the RAS superfamily act as molecular switches in the transduction of cellular signals critical for a wide range of normal developmental events as well as pathological processes. However, the functions of *Ras* genes in ovarian cells have only started to be unveiled. RAS, most likely KRAS that is highly expressed in granulosa cells of growing follicles, appears crucial for mediating the gonadotropin-induced events associated with the unique physiological process of ovulation. By contrast, conditional expression of a constitutively active *Kras*^{G12D} mutant in granulosa cells results in ovulation defects due to the complete disruption of normal follicular growth, cessation of granulosa cell proliferation, and blockage of granulosa cell apoptosis and differentiation. When the tumor suppressor *Pten* is disrupted conditionally in the *Kras*^{G12D}-expressing granulosa cells, granulosa cell tumors fail to develop. However, ovarian surface epithelial cells expressing the same *Pten*;*Kras*^{G12D} mutations rapidly become ovarian surface epithelial serous cystadenocarcinomas. In this minireview, we summarize some of the physiological as well as pathological functions of RAS in the rodent ovary, discuss the implications of the *Kras*^{G12D} mutant mouse models for understanding human diseases such as premature ovarian failure and ovarian cancers, and highlight new questions raised by the results of recent studies. **(*Molecular Endocrinology* 24: 286–298, 2010)**

RAS (rat sarcoma viral oncogene) proteins function as molecular switches regulated at the level of GDP/GTP binding and play critical roles in controlling normal cellular proliferation as well as in the development of neoplasia. Activating mutations in the *Ras* gene family members are found in 30% of all human tumors (1). In mammals, there are three functional *Ras* genes, *H*(*Harvey*)*ras*, *N*(*neuroblastoma*)*ras*, and *K*(*Kirsten*)*ras*, located on different chromosomes that appear to be expressed ubiquitously (2). In addition, genes encoding three other small G proteins, *R*(*related*)*ras*, *Rras2* (TC21), and *M*(*muscle*)*ras* are structurally and functionally related members of this family (3) and can impact cell motility, cell adhesion, and transformation (4–6). The first 86 amino acids of the mammalian HRAS, NRAS, and KRAS proteins, which harbor the putative effector domain, are 100% identical.

Despite the diverse functions of these RAS proteins in various signal transduction events, *Nras* and *Hras* are nonessential for mouse development. Mice that are homozygous null for either *Nras* (7) or *Hras* (8) are viable and exhibit no overt developmental or postnatal abnormalities. However, embryos homozygous null for *Kras* die between 12 and 14 d of gestation, with liver defects and evidence of anemia, suggesting that *Kras* is the only *Ras* family member that is crucial for embryonic development and cannot be compensated for by other *Ras* genes, assuming that they are expressed at these early embryonic stages (9). RAS proteins transduce signals through different effectors [*e.g.* Ras-activated factor (serine/threonine kinase), phosphatidylinositol 3-kinase (PI3K), RAL-guanine nucleotide dissociation stimulator (GDS), and RAS-guanine nucleotide release protein (GAP)], which regulate diverse cell functions. They become oncogenic by single point muta-

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Abbreviations: AREG, Amphiregulin; C/EBPβ, CAAT/enhancer binding protein-β; COC, cumulus cell-oocyte complex; eCG, equine chorionic gonadotropin; EGF, epidermal growth factor; EGFR, EGF receptor; FOXO, Forkhead box O; GCT, granulosa cell tumor; GPCR, Gs protein-coupled receptor; hCG, human chorionic gonadotropin; MEK, MAPK kinase; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; POF, premature ovarian failure; PTEN, phosphatase with tensin homolog.

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tions, mainly at either codon 12 or 13, which lead to constitutive signaling and cell transformation associated with changes in cell morphology, increased proliferation, and/or inhibition of apoptosis (10–12). RAS proteins can also induce cell senescence, most frequently associated with epithelial cells and fibroblasts (13–15).

Despite the extensive involvement of RAS factors in activating normal signal transduction and aberrant tumorigenesis of eukaryotic cells, the potential roles of RAS in mediating the effects of hormones and growth factors in ovarian granulosa cells (16, 17) and the physiological functions of *Ras* in the ovary have only started to be unraveled (18). The embryonic lethality of *Kras* knockout mice and the inconvenience of primary ovarian cells as tools for biochemical studies have prevented the study of *Ras* with respect to mammalian reproductive biology *in vivo*. However, the recent development of mouse strains carrying *Ras* conditional mutant alleles and tissue-specific expressed Cre recombinase have permitted us to investigate the roles of KRAS in specific ovarian cell types (18, 19). In this minireview, we summarize recent studies of the physiological and pathological functions of RAS in the rodent ovary and discuss how the *Ras* mutant mouse models may provide models to help understand human diseases such as premature ovarian failure (POF) and ovarian cancers.

Physiological Functions of RAS in the Rodent Ovary

The mammalian ovary controls female fertility by regulating the continuous growth and timely maturation of follicles destined to ovulate and release a fertilizable oocyte (egg). Formation of the progesterone-producing corpus luteum after ovulation is requisite for successful initiation of pregnancy. Moreover, within growing follicles, it is now clear that the oocyte and its surrounding somatic cells, the cumulus cells, comprise a unique microenvironment. Before ovulation, the cumulus cell-oocyte complex undergoes dramatic changes that are required for successful ovulation and oocyte maturation. Collectively, these ovarian events are regulated by the coordinate production and action of local ovarian regulatory factors (steroids and proteins) and the pituitary gonadotropins, FSH and LH (20–22). FSH and LH bind their cognate seven-transmembrane, Gs protein-coupled receptors (GPCRs), FSH receptor, and LH chorionic gonadotropin receptor, respectively, and thereby increase intracellular cAMP and activate protein kinase A (PKA) (23). 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 via their receptors in ovarian granulosa cells are more complex. Some molecular events, such as the phosphorylation of protein kinase B (PKB/AKT) and MAPKs (also known as ERKs), can occur independently of PKA activation (16, 23, 24). The FSH and LH receptors, like other GPCRs, can activate RAS and specific tyrosine kinase cascades, a pattern that has been established for GPCRs in other cells (25–27). The facts that LH via its receptor can activate RAS in an MA10 mouse Leydig cell line and that activation of RAS induces phosphorylation of ERK1/2 via a receptor tyrosine kinase-dependent mechanism (28) provide support for the notion that gonadotropin receptors are also linked functionally to RAS and tyrosine kinase activities.

Although LH directly stimulates functional changes in theca cells and granulosa cells of preovulatory follicles, its effects on cumulus cells and oocytes are probably indirect. In mice, these cells, in contrast to granulosa cells, express few or no LH receptors (LH chorionic gonadotropin receptors) and fail to respond when directly stimulated by LH in culture (29). In recent years, members of the epidermal growth factor (EGF)-like growth factor family have emerged as likely mediators of LH action in the preovulatory follicle. Specifically, the pioneering studies of Espey, Conti, and others (29–33) have shown that amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC) are induced rapidly by LH or its analog human chorionic gonadotropin (hCG) in granulosa cells and cumulus cells. These factors are thought to function by both autocrine and paracrine mechanisms to propagate LH signals throughout the preovulatory follicle to induce ovulation, cumulus cell-oocyte complex (COC) expansion, oocyte maturation, and luteinization (29, 31, 32). The growth factor effects require the EGF receptor (EGFR, also called ERBB1), because EGFR tyrosine kinase inhibitors and disruption of the EGF signaling network *in vivo* block COC expansion and ovulation (31, 32).

Recently and of physiological relevance, activation of RAS in granulosa cells by the gonadotropins has also been documented *in vivo*. In the mouse ovary, KRAS is highly expressed in granulosa cells of small and large growing follicles and at lower levels in luteal cells (18) (Fig. 1A). During hormone-induced ovulation, the levels of total RAS do not change. However, the levels of active, GTP-bound RAS that are undetectable in ovaries of immature mice, increased slightly in response to the FSH-like gonadotropin equine chorionic gonadotropin (eCG) that stimulates preovulatory follicle development. Activated RAS was increased markedly (but transiently) at 2 h after hCG treatment that stimulates ovulation. Notably, hCG also induces significant phosphorylation of the EGFR, MAPK kinase (MEK)-1/2, and ERK1/2, all maximal at

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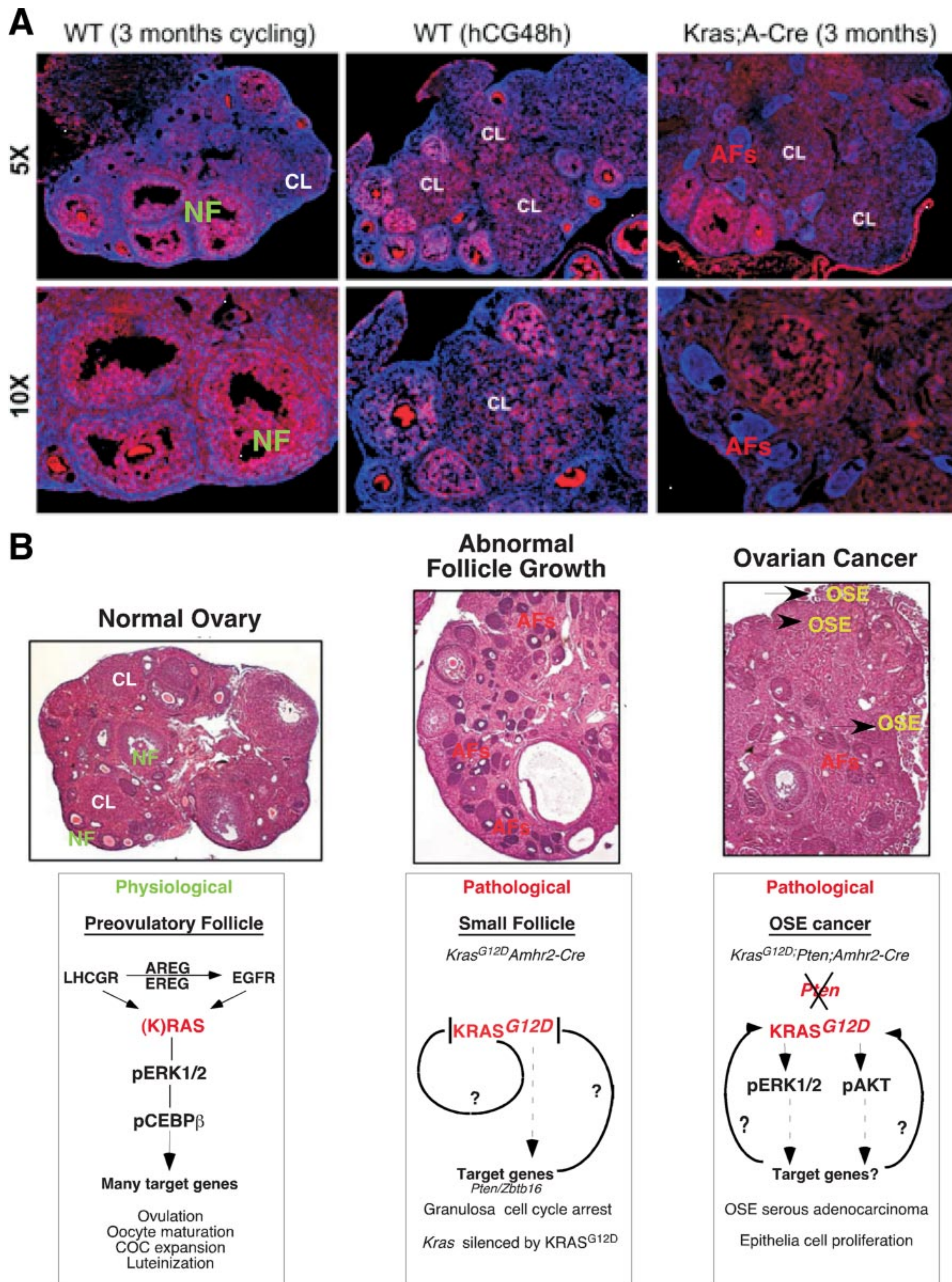

FIG. 1. KRAS expression and functions in ovarian cells. A, Immunocytochemical staining shows that KRAS protein is expressed at high levels in granulosa cells of normal growing follicles (NF) of wild-type (WT) adult mice (3 months of age) but is totally absent from cells in the abnormal follicles (AFs) present in ovaries of the *Kras*^{G12D};*Amhr2-Cre* mutant mice (3 months of age). KRAS is also present in corpora lutea (CL) of WT mice (3 months of age) and in hormonally primed immature mice 48 h after hCG. KRAS is also present in oocytes. B, Ovarian histology and functional outcomes of RAS activation during physiological and pathological conditions. In the normal ovary, LH induction of ovulation and luteinization is dependent on the activation of RAS and the downstream kinases ERK1/2. Premature exposure of granulosa cells to constitutively active KRAS^{G12D} leads to the formation of abnormal follicles in which the granulosa cells cease to divide, are nonapoptotic, and fail to express markers of granulosa cell differentiation. Of note, the *Kras* gene is silenced in the cells within the abnormal follicles (A), but they express high levels of PTEN and *Zbtb16* (*Pzlf*) (19). Mice in which *Kras*^{G12D} is expressed in granulosa cells lacking *Pten* (*Kras*^{G12D};*Pten*$^{-/-}$;*Amhr2-Cre* mutant mice), abnormal follicles but no granulosa cell tumors form. By contrast, OSE cells are highly proliferative, and adenocarcinomas appear rapidly in the *Kras*^{G12D};*Pten*$^{-/-}$;*Amhr2-Cre* mutant mice (19). pERK1/2 phospho-ERK1/2.

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2 h after hCG (18, 34). Activated RAS-GTP in ovarian granulosa cells can be pulled down by the RAF1 RAS-binding motif fused to a glutathione S-transferase tag, indicating the direct impact of RAS on the canonical RAF1/MEK/MAPK cascade in these cells *in vivo* (18). In addition, undifferentiated granulosa cells isolated from small follicles of estrogen-primed immature rats respond in culture to both FSH and AREG and exhibit rapid phosphorylation/activation of ERK1/2, p38MAPK, and AKT, demonstrating the branched signaling events can be initiated in granulosa cells at early stages of follicle growth as well (16). Importantly, RAS-GTP levels are also transiently increased after FSH or AREG stimulation, suggesting that RAS activation is likely involved. As further evidence, overexpression of a dominant-negative RAS mutant blocks both MAPK (including ERK1/2 and p38MAPK) phosphorylation and granulosa cell differentiation to luteal-like cells induced by FSH and AREG in culture. However, activation of the PI3K pathway in granulosa cells cannot be blocked by dominant-negative RAS but is sensitive to inhibitors of rous sarcoma oncogene (SRC) family tyrosine kinases (13). Therefore, both *in vivo* and *in vitro* studies indicate that RAS, most likely KRAS, is an important physiological mediator of gonadotropin signaling in ovarian follicles.

More recently, ERK1/2, the downstream target kinases of the EGFR/RAS pathway have been disrupted in granulosa cells, and these mutations cause dramatic changes in granulosa cell fate. Although follicular development is normal in the *Erk1/2* mutant mice, they are completely infertile because ovulation, COC expansion, and oocyte maturation as well as luteinization are all blocked (34). The global 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* (32, 34, 35). Additionally, the transcription factor CAAT/enhancer binding protein-β (C/EBPβ) is a target of ERK1/2 and appears to control some of the cell fate decisions in granulosa cells of ovulatory follicles (34, 36). This is most likely associated with cell cycle arrest based on the impact of C/EBPβ in other endocrine tissues such as uterine stromal cells (37) and adipocytes (38) as well as its connections with senescence in fibroblasts (39, 40). Interestingly, cell cycle arrest in each of these cell types appears to involve the production of potent cytokines such as IL-6 (37, 38, 40, 41). 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 that SF1, is a potential RAS-ERK1/2 target in granulosa cells of preovulatory follicles. Specifically, mice in which the *Lrh1* gene is conditionally disrupted in granulosa cells show ovulation and luteinization defects (42), whereas conditional disruption of *Nr5a1* in granulosa cells selectively impairs early stages of follicle growth (43). In addition, LRH1 is known to be phosphorylated and functionally activated by ERK1/2 (44).

Therefore, RAS, most likely KRAS, is an important physiological mediator of gonadotropin signaling in ovarian follicles, especially at the time of ovulation when activation of ERK1/2 is essential not only for follicle rupture but also for oocyte meiotic maturation, COC expansion, and luteinization (34, 45). Thus, at the preovulatory stage of follicular growth, RAS and ERK1/2 activation are obligatory to direct the terminal differentiation of granulosa cells to nondividing luteal cells (34) (Fig. 1, A and B). Because follicular growth was normal in mice in which ERK1/2 were depleted in granulosa cells and because the activation of RAS in granulosa cells was lower in response to FSH/eCG *in vivo*, one might predict that activated RAS and ERK1/2 are not essential and might even exert pathological effects at earlier stages of follicle growth.

Granulosa Cell-Specific *Kras*^{G12D} Mutation Causes Stage-Dependent Ovarian Defects

The effects of *Ras* gene mutations have been studied in primary and transformed cell lines as well as in transgenic mice (11, 46). However, traditional mutation strategies direct expression of the oncogenes to specific cells of the target tissue based on promoter activity and may lead to supraphysiological levels of expression. In an effort to overcome these limitations, Tyler Jacks and colleagues (47, 48) have created new mouse strains harboring latent, oncogenic alleles of *Kras* and *Nras* capable of tissue- and cell type-specific activation *in vivo*, triggered by CRE recombinase-mediated DNA recombination. Mice expressing a germline *Kras*^{G12D} mutation exhibit early embryonic lethality due to a placental trophoblast defect (49). Mutant embryos demonstrate cardiovascular and hematopoietic defects as well as a profound defect in lung branching morphogenesis associated with up-regulation of the MAPK antagonist *Sprouty-2* and abnormal activation of MAPK within the lung epithelium (50).

To analyze the functions of RAS protein in ovarian cells *in vivo*, several mouse models have been generated (Table 1). Orsulic *et al.* (51) introduced mutations in ovarian cells by combining *in vitro* and *in vivo* approaches. Specifically, potential oncogenic factors (*K-ras*, *c-myc*, or *Akt*) were introduced via replication-competent avian leukosis virus-derived (RCAS) vectors into p53$^{-/-}$ mouse ovarian cells expressing the avian retroviral recep-

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TABLE 1. Selective ovarian-specific mutant mouse models that exhibit altered follicular development, granulosa cell tumors, or OSE cell tumors: distinct responses of granulosa cells to oncogenes

Genotype	Ovarian phenotypes	Ovarian tumor type	Refs.
Kras^{G12D};*Amhr2–Cre* and *Kras*^{G12D};*Cyp19–Cre*	Decreased ovulation; arrest of GC proliferation and differentiation; accumulation of abnormal follicles; premature ovarian failure	None	18
Kras^{G12D};*Pgr–Cre* and *Pten*$^{-fl/fl}$; *Kras*^{G12D};*Pgr–Cre*	No overt ovarian phenotypes	None	19
Erk1$^{-/-}$;*Erk2*$^{-fl/fl}$;*Cyp19–Cre*	No ovulation or luteinization in response to LH/hCG; Prolonged follicular development	None	34
Pten$^{-fl/fl}$; *Amhr2–Cre*	Accumulation of corpora lutea; ovarian stromal hyperplasia	Rare GCT (10%) in aged mice	75
Pten$^{-fl/fl}$; *Cyp19–Cre*	Accumulation of corpora lutea; increased GC proliferation and ovulation; decreased follicle atresia	None	58
Pten$^{-fl/fl}$; *Kras*^{G12D};*Amhr2–Cre*	Ovulation failure; arrest of GC proliferation and differentiation; OSE tumors by 2 months of age; die at 3–6 months of age	100% OSE tumor after 3 months old	19
Pten$^{-fl/fl}$; *Kras*^{G12D};*Cyp19–Cre*	Ovulation failure; arrest of GC proliferation and differentiation; premature ovarian failure; no tumors	None	19
Ctnnb1$^{Ex3fl/+}$;*Amhr2–Cre*	Reduced ovulation; subfertile; pretumor ovarian lesions in mice 1–6 months of age	100% GCT in aged mice	69, 70
Pten$^{-fl/fl}$;*Ctnnb1*$^{Ex3fl/+}$;*Amhr2–Cre*	Aggressive GCTs leading to death at a young age	100% GCT in new born mice	75
Inha$^{-/-}$	Infertile; GCTs	100% GCT after 3 months old	66
Smad1$^{fl/-}$;*Smad5*$^{fl/-}$;*Amhr2–Cre*	Subfertile; GCTs	100% GCT after 3 months old	68
Pten$^{-fl/fl}$;*APC*$^{-fl/fl}$ injected with Ad-Cre	Not reported	100% OSE tumors	54
Pten$^{-fl/fl}$; *Kras*^{G12D} injected with Ad-Cre	Not reported	100% OSE tumors	53
P53$^{-/-}$;*Tg–c–Myc/Kras* and *P53*$^{-/-}$;*Tg–Akt/Kras*	Not reported	100% OSE tumors	51
Tg–Amhr2–TAg	Not reported	50% OSE tumors	52
bLHβ transgenic mice	Ovulation infrequent; prolonged luteal phase	40% GCTs and luteomas 4–8 months of age	78

tor, TVA. When transplanted into wild-type mice, these mutant cells formed tumors if two or more oncogenes were coordinately expressed. These studies provided the first documentation that ovarian surface epithelial cells, and not gonadal mesenchymal cells, are the likely cellular source for ovarian carcinomas in humans. However, the model does not lend itself easily to analyzing the etiology of cancer. In another important contribution, Connolly *et al.* (52) generated mutant mice in which the *Amhr2* promoter was used to drive expression of the simian virus 40 T antigen. These mutant mice developed ovarian tumors and by specific markers were shown to be derived from ovarian surface epithelial (OSE) cells, not granulosa cells. Therefore, these mice provided the first evidence that *Amhr2* could be expressed in OSE cells. These mice have been invaluable not only for *in vivo* analyses but also for the generation of transformed cell lines that are being used by many investigators (52). However, the use of the simian virus 40 does not necessarily recapitulate the specific mutation of genes altered in human ovarian cancers. Adding further proof that OSE cells are the likely cellular source for most ovarian cancers, Dinulescu *et al.* (53) injected adenoviral vectors expressing Cre recombinase under the ovarian bursa of *Pten*$^{fl/fl}$; *Loxp-stop-Loxp* (*LSL*)-*Kras*^{G12D} mice. These mice developed endometrioid-like ovarian cancer in which the epithelial cells (cytokeratin 8 positive) expressed elevated levels of phospho-AKT and phospho-ERK. This same adenoviral Cre approach has been used to generate OSE cancers in *Pten*$^{fl/fl}$;*Apc*$^{fl/fl}$ mice (54). The pioneering studies of Donahoe and colleagues (55) who have reported that anti-Mullerian hormone (AMH) [also called Mullerian-inhibiting substance (MIS)] can block proliferation of ovarian cancer cells in culture provide additional evidence that ovarian epithelial cells express AMH receptor 2, that this signaling pathway impacts proliferation, and that OSE cells are the source of ovarian carcinomas. Collectively, these studies have helped to dispel the notion held by some that *Amhr2*

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is not expressed or functional in OSE cells. Thus, there is increasing evidence that some human (and mouse) ovarian carcinomas are derived from the surface epithelial cells of the ovary and that these cells express AMH receptor 2. Furthermore, these results provide strong evidence that granulosa cells that also express the *Amhr2* escape oncogenic transformation in the studies by Orsulic *et al.* (51) and Connolly *et al.* (52). Moreover, we have mutant mouse models that reinforce the notion that granulosa cells respond differently to *Kras* mutations than do OSE cells and also provide the first *in vivo* mouse model in which OSE cancer develops spontaneously in response to cell-specific expression of Cre.

Specifically, we generated conditional knock-in mouse models, using the *Loxp-Stop-Loxp Kras*^{G12D} mouse strain (47, 49) and selected Cre strains in which the granulosa cells express constitutively active *Kras*^{G12D} at specific stages of follicular growth (18) (Table 1). As already mentioned, *Amhr2-Cre* is expressed in granulosa cells of small and growing follicles as well as OSE cells, but the efficiency of DNA excision of target genes varies among the published reports (19, 56, 57). By contrast, *Cyp19-Cre* is highest in granulosa cells of antral and preovulatory follicles but is absent from OSE cells (18, 58). Although *Cyp19-Cre* is expressed in a subset of granulosa cells in preantral and early antral follicles, the Cre transgene is highly expressed in all the granulosa cells of preovulatory follicles and induces 90–100% DNA excision of target genes (18, 58). Because the progesterone receptor (*Pgr*) is expressed only in granulosa cells of preovulatory follicles exposed to the LH surge, Cre recombinase is active only in luteinizing granulosa cells after the LH surge in the *Pgr-Cre* mice (59). The *Kras*^{G12D};*Amhr2-Cre* and *Kras*^{G12D};*Cyp19-Cre* conditional mutant mice were subfertile in the first 3 months of age and became completely infertile thereafter (18). Further studies demonstrated that the adult *Kras*^{G12D} mutant mice have few healthy growing follicles in their ovaries and very low levels of estrogen but high levels of serum gonadotropins. Thus, the mice exhibit the symptoms similar to POF in human patients. More surprisingly, the ovaries of these *Kras*^{G12D};*Amhr2-Cre* and *Kras*^{G12D};*Cyp19-Cre* mice contain many small abnormal follicle-like structures in which the granulosa cells are nonmitotic and nonapoptotic and fail to express known markers of granulosa cell differentiation, including FSH and LH receptors (18) (Fig. 1, A and B). 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. Importantly, expression of KRAS^{G12D} in granulosa cells silenced the endogenous *Kras* gene such that neither *Kras* mRNA nor protein could be detected in the abnormal cells. As a consequence, expression of KRAS^{G12D} in granulosa cells did not stimulate granulosa cell proliferation (Fig. 1A).

In mice that carry the granulosa cell-specific *Kras*^{G12D} mutations (induced by *Amhr2-Cre* or *Cyp19-Cre*), the few follicles that are not arrested at an early stage of development proceed to the antral stage. However, these *Kras*^{G12D} antral follicles fail to ovulate and express reduced levels of ovulation-related genes, such as *Areg*, *Ptgs2*, *Has2*, and *Tnfaip6* (18). Consequently, two prerequisites of successful ovulation, meiotic resumption in oocytes and expansion of COCs, are compromised in the ovaries of the *Kras* mutant mice. Not surprisingly, the number of oocytes being ovulated was reduced markedly in these animals. However, the granulosa cells luteinize and form corpora lutea although with abnormal looking histology and entrapped oocytes. Interestingly, oocytes retrieved from the few preovulatory follicles that develop in the eCG-primed *Kras* mutant mice are able to undergo spontaneous meiotic maturation in a manner similar to those isolated from wild-type mice. The mature eggs occasionally being ovulated by *Kras* mutant mice can be fertilized and undergo normal embryonic development (Fan, H.-Y., and J. S. Richards, unpublished observations). In addition to these defects observed *in vivo*, when *Kras*^{G12D} was expressed in cultured cells using adenoviral Cre approaches, the expression of FSH and LH receptors (*Fshr* and *Lhcgr*) was decreased, and the response of the cells to ovulatory signals was blocked (18). Collectively, these observations indicate that the observed effects of *Kras*^{G12D} on ovarian follicles are context and granulosa cell specific. That is, expression of KRAS^{G12D} in small follicles causes growth arrest, whereas expression at later stages of follicle development impairs ovulation but not oocyte functions (19). If expressed after the LH surge as in the *Pgr-Cre* mice, KRAS^{G12D} appears to exert no overt effects (19).

The eCG- and hCG-stimulated phosphorylation of ERK1/2 observed in granulosa cells of ovulatory follicles of wild-type mice was reduced markedly in *Kras*^{G12D} mutant granulosa cells (18). Reduced ERK1/2 phosphorylation was due, in part, to increased expression of MAPK phosphatase MKP3 [also known as duel-specific phosphatase 6 (DUSP6)], an ERK1/2-specific phosphatase. *Kras*^{G12D} expression in granulosa cells leads to the upregulation of *Mkp3*/*Dusp6* levels both *in vivo* and *in vitro*. Interestingly, in our previous studies, *Dusp6* was also identified as a gene being induced dramatically in mouse granulosa/cumulus cells by LH surge (30). In the eCG/hCG-induced ovulation model, the maximal expression of *Dusp6* was observed at 4 h after hCG treatment, when the transient preovulatory phosphorylation of

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ERK1/2 is terminated. Depletion of *Dusp6* in granulosa cells by RNA interference prolonged the AREG-induced ERK1/2 phosphorylation in culture. In addition, ERK1/2 apparently induces the expression of *Dusp6*, because the MEK1/2 inhibitor U0126 blocks AREG-induced *Dusp6* expression in cultured granulosa cells, and hCG treatment fails to induce *Dusp6* expression in ovaries from mice carrying granulosa cell-specific mutations of ERK1/2 (18, 34). Expression levels of *Sprouty*-2 (as well as two other mammalian *Sprouty* family members, *Spry 1* and *4*), a MAPK antagonist that is selectively up-regulated by KRAS^{G12D} in mouse lung epithelium (50), do not change in *Kras* mutant granulosa cells (Fan, H.-Y., and J. S. Richards, unpublished observations). These results suggest that although KRAS^{G12D} attenuates the activity of MAPK cascade in both developing lung branches and ovarian follicles, it achieves these effects by inducing different antagonists of this pathway. Collectively, these data indicate that *Dusp6* is one negative feedback regulator of ERK1/2 activity, being induced in both physiological conditions and *Kras* mutant conditions.

Kras Mutation and Cell Cycle Arrest in Granulosa Cells

As mentioned above, *Kras*^{G12D} expression in granulosa cells at early (secondary/preantral) stages of follicle growth induces the formation of many abnormal follicle-like structures in which the granulosa cells are nonmitotic, nonapoptotic, and poorly differentiated (18) (Fig. 1, A and B). That the formation of these abnormal follicle-like structures is dependent on the stage of granulosa cell differentiation and follicle development (18, 19) was demonstrated by their presence in the ovaries of *Kras*^{G12D};*Amhr2-Cre* and *Kras*^{G12D};*Cyp19-Cre* mice where recombinase is expressed in granulosa cells of small growing follicles. By contrast, the abnormal follicles were not observed in the *Kras*^{G12D};*Pgr-Cre* mutant mice where recombination occurs only after the LH surge (19). 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.

Overexpression of oncogenic *Kras* mutants not only renders cells susceptible to tumorigenesis but can also induce premature senescence in some primary cell cultures as well as in some cells, most notably epithelial cells and fibroblasts, *in vivo* (13, 46). In these cell types, the senescent response is associated with, and regulated by, increased levels of cell cycle inhibitors such as p15^{INK4B}, p16^{INK4A}, and the tumor repressor p53 (13, 46, 60). Senescence in some cell types appears to be dependent on ERK1/2 and ribosomal S6 kinase activation of C/EBPβ (61). It is also associated with a highly predictable and elevated pattern of secreted cytokines, such as IL-6 (40, 62). However, it appears that the molecular factors causing cell cycle arrest in granulosa cells within the abnormal follicle lesions found in *Kras*^{G12D};*Amhr2-Cre* mice may differ from those driving senescence in epithelial cells and fibroblasts. For example, neither Western blot analyses nor immunostaining detected increased expression of p15^{INK4B}, p16^{INK4A}, p53, or C/EBPβ (unpublished observation) in *Kras* mutant ovaries (19). However, overexpression of KRAS^{G12D}, unlike endogenous KRAS^{G12D}, is able to up-regulate the protein levels of p15^{INK4B}, p16^{INK4A}, and p53 and induce cell cycle arrest in cultured granulosa cells (19). These results suggest that endogenous levels of oncogenic *Kras* likely induce cell cycle arrest of granulosa cell by a mechanism that is distinct and apparently independent of the known p15^{INK4B}/p16^{INK4A}/p53 pathway. However, the canonical cell senescence pathway induced by high levels of oncogenic *Ras* mutations is conserved in granulosa cells.

Although the detailed mechanisms by which endogenous levels of *Kras*^{G12D} lead to cell cycle arrest of granulosa cells remain unknown, recent results indicate that it involves the rapid silencing of *Kras* gene itself (mediated by activated ERK1/2 and/or C/EBPβ?) and the up-regulation of tumor repressor *Pten*. Loss of KRAS and increased phosphatase with tensin homolog (PTEN) lead secondarily to the absence of both phospho-ERK1/2 and phospho-AKT in the mutant cells (19) (Fig. 1B). In addition, the *Kras*^{G12D}-expressing granulosa cells exhibit markedly reduced levels of the cell cycle activators cyclin A, cyclin D2, and E2F1 and increased levels of *Zbtb16* (*Plzf*), a regulator of cell cycle arrest. Because activation of 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 (19).

Pathological Effects of *Ras* Mutations and Ovarian Cancer

As indicated above, about 30% of human tumors carry *Ras* gene mutations (1). Of the oncogenic genes in this family, *Kras* is the most frequently mutated member in human tumors, including adenocarcinomas of the pancreas (~70–90% incidence) (10), colon (~50%) (48), and lung (~25–50%) (47). The *Kras*^{G12D} conditional knock-in mouse model has been extensively employed to study the mechanisms of *Ras*-induced tumor develop-

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ment (47, 50, 53). The conditional expression of *Kras*^{G12D} in mice, when combined with other mutations, leads to malignant tumorigenesis in various tissues, including lung, mammary gland, OSE, and uterus (46, 50, 53) (Wang, J., and F. J. DeMayo, personal communications). The responses of cells to RAS activation appear to be context, developmental, and strength specific such that cells may either undergo oncogenic transformation or become senescent (46). In the ovary, it is now clear that granulosa cells respond to *Kras*^{G12D} mutant protein and undergo cell cycle arrest, whereas the surface epithelial cells respond to the same mutation and become hyperproliferative.

OSE cancers make up nearly 90% of all ovarian cancers. In contrast, only 5% of human ovarian cancers are derived from granulosa cells (63, 64). 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 nondividing 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 mutation of *Foxl2*, a transcription factor important for early follicle development and granulosa cell proliferation, that has been associated with human adult GCTs (65). Known mutant mouse models exhibiting high frequencies of granulosa tumor development include inhibin-α-null mice (66, 67), granulosa cell-specific *Smad1/5/8* knockout mice (68), and granulosa cell-specific β-catenin mutant mice (69, 70). None of these models appears to involve RAS-regulated signaling events. Because most cancer cells express more than one mutant gene, numerous studies, including those in ovarian cells, have been done to determine the effects of different combinations of oncogenic factors, caused by both activating as well as inactivating mutations (64).

The tumor suppressor PTEN is often disrupted in many tumor cells (71, 72). As a potent inhibitor of the PI3K pathway, PTEN impacts the phosphorylation and functional status of several factors, including, AKT and Forkhead box O (FOXO) factors. Because FOXO1 is elevated in granulosa cells of growing follicles (73, 74), we generated conditional disruption of the *Pten* gene in granulosa cells using the *Amhr2-Cre* and *Cyp19-Cre* mouse strains. Mice in which *Pten* alone was disrupted in granulosa cells develop GCTs but with a very low frequency (75). They do not develop OSE tumors. Rather, the *Pten*$^{fl/fl}$;*Cyp19-Cre* and *Pten*$^{fl/fl}$;*Amhr2-Cre* mice exhibit enhanced ovulation, reduced apoptosis (associated with reduced levels of FOXO1), and contain corpora lutea with an extended life span (58). By contrast, when *Pten* is disrupted in the *Kras* mutant strain of mice, the *Pten*$^{fl/fl}$;*Kras*^{G12D};*Amhr2-Cre* but not the *Pten*$^{fl/fl}$;*Kras*^{G12D};*Cyp19-Cre* double-mutant mice develop OSE tumors. The specificity of OSE tumor formation is due to the expression of the *Amhr2* but not *Cyp19* in OSE cells (19). The epithelial cells of these tumors express cytokeratin 8, WT1, and MUC16, suggesting that they are serous adenocarcinomas (76). As might be expected, the mutant cells exhibit elevated activities of the ERK1/2 pathway and PI3K/AKT pathways. Strikingly, the *Pten*$^{fl/fl}$;*Kras*^{G12D} double-mutant mice do not develop 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 (19). That the *Kras*^{G12D}/*Pten* mutant granulosa cells do not undergo tumorigenic transformation is quite unique and indicates that these cells are extremely resistant to this specific combination of oncogenic insults. 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. Thus, within the ovaries of the same *Kras*^{G12D}/*Pten* mutant mouse, granulosa cells exit the cell cycle, whereas the OSE becomes hyperplastic. Therefore, the *Kras*^{G12D}/*Pten* double-mutant mice afford a unique model in which to track two opposing cell cycle forces, cell cycle arrest *vs.* proliferation, and to determine why early granulosa cells and OSE cells are susceptible to their dramatically altered fates and what cell signaling cascades, genes, and/or epigenetic factors downstream of KRAS dictate these effects.

Contrary to the lack of granulosa cell tumors (GCTs) in *Pten*$^{fl/fl}$;*Amhr2-Cre* or *Kras*^{G12D};*Amhr2-Cre* mutant mice, mice in which *Smad1/5/8* (68) or *Inha* (encoding inhibin 2) (66) are disrupted develop GCTs with 100% penetrance. In addition, mice that express dominant active β-catenin alone in granulosa cells develop GCTs. The recombined *Ctnnb1*$^{flox(ex3)}$ allele encodes a β-catenin protein that, although still functional, lacks a series of phosphorylation sites that are required for its degradation, resulting in its inappropriate accumulation and translocation to the nucleus. Interestingly, *Ctnnb1*$^{flox(ex3)/+}$;*Amhr2-Cre* mice developed pretumor follicle lesions, which consist of follicle-like nests of disorganized, pleiomorphic granulosa cells. These pretumor lesions grow no larger than the size of antral follicles but often evolved into GCT in older mice. These data showed a causal link between misregulated Wnt/β-catenin signaling and GCT development (69, 70). Although the pretumor follicle lesions of *Ctnnb1* mutant mice share some morphological similarities to the

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abnormal follicle-like structures present in ovaries of *Kras*^{G12D} mutant mice, the genes expressed in the granulosa cells of each genotype differ markedly. Whereas negative regulators of the WNT signaling pathway, *Axin2* and *Nkd1*, were elevated in the *Ctnnb1*-expressing cells (70), neither of these genes was induced in the *Kras* mutant granulosa cells. Conversely, PTEN is not elevated in the *Ctnnb1* mutant cells but is elevated in the *Kras* mutant cells. Thus, KRAS^{G12D} and β-catenin$^{\Delta ex3}$ regulate distinct processes in granulosa cells, leading to cell cycle arrest in the follicle lesions and GCTs, respectively. By comparing the *Kras* and β-catenin mutant granulosa cells at cellular and molecular levels, we should be able to obtain deeper insights into the mechanisms leading to granulosa cell cycle arrest and tumorigenesis. Furthermore, to determine the relationship between RAS-related signaling events and the WNT pathway, it will be interesting to determine the ovarian phenotype of *Kras*; *Ctnnb1* double-mutant mice, especially to test whether the mutant *Kras* can repress or facilitate the β-catenin$^{\Delta ex3}$-induced GCTs.

Clinical Implications

Based on the foregoing review, it is clear that the role of RAS, most likely KRAS, in the ovary is cell context specific. Moreover, RAS activation is tightly regulated in granulosa cells and OSE cells. Whereas the activation of RAS is critical for initiating LH-mediated events, such as ERK1/2 phosphorylation, that are essential for ovulation and luteinization, inappropriate expression of a mutant constitutively active form of KRAS (KRAS^{G12D}) leads to granulosa cell cycle arrest and impaired follicle growth. By contrast, expression of KRAS^{G12D} in *Pten*-deficient OSE cells leads to serous epithelial adenocarcinomas. Thus, mutations in RAS could be associated with some unknown causes of POF as well as ovarian cancer. In addition, because POF is often associated with elevated levels of FSH and because FSH can activate RAS in granulosa cells in culture and i*n vivo* (albeit to a lesser extent than LH), it is possible that prolonged stimulation of granulosa cells by FSH could also inappropriately activates RAS, thereby impairing their functions and causing cell cycle arrest. Importantly, prolonged exposure of mouse ovaries to FSH does not lead to ovarian cancer (77). RAS may be involved in polycystic ovarian syndrome where levels of LH but not FSH are elevated. In this scenario, RAS may be activated inappropriately in theca cells. Chronic levels of LH have also been shown to cause benign ovarian luteomas in mice (78); hence, similar effects may impact ovarian cells in postmenopausal 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. Thus, the most important physiological function of RAS in the gonads is its activation at the time of ovulation. In this regard, it is important to note that induction of the LH surge is an ERK1/2-dependent event that, although not yet known, is likely a RAS-dependent event as well (79). ERK1/2 is not critical for basal LH secretion in male mice. The most important pathological effects of RAS activation in the ovary appears to be the potent effect of mutant RAS to cause cell cycle arrest in granulosa cells and facilitate OSE cancer.

Future Directions

Although many factors that control cell cycle progression and transformation have been identified and their functions characterized, less is known about what causes cells to exit the cell cycle and become quiescent, senescent, or terminally differentiated. If the factors that force cells to exit the cell cycle irreversibly were known and their functions understood, these factors might be harnessed to provide new avenues, reagents, and approaches to block cancer cell proliferation. We hypothesize that specific regulatory molecules, in response to the activation of the endogenous RAS (KRAS) in granulosa cells of preovulatory follicles, cause the transformation of these proliferating granulosa cells to nondividing terminally differentiated luteal cells. Premature expression of the oncogenic *Kras*^{G12D} mutant in granulosa cells of small follicles dictates their premature exit from the cell cycle and renders them uniquely impervious to insults by specific oncogenic factors.

Many Questions Arise

What factors impede the potential oncogenic functions of RAS in granulosa cells and direct the cells to exit the cell cycle? Are the cell cycle-arrested granulosa cells present in the abnormal follicle-like structures quiescent, senescent, or prematurely terminally differentiated? Granulosa cells of preovulatory follicles respond to activation of RAS, ERK1/2, and C/EBPβ and cease dividing and become terminally differentiated luteal cells. Therefore, it is tempting to speculate that granulosa cells of small follicles expressing of KRAS^{G12D} respond to the same factors in a similar manner. But are they terminally differentiated? The answer is probably no, because with the exception of PTEN, the cell cycle-arrested granulosa cells present in the abnormal follicles do not express known granulosa cell or luteal cell markers (12, 28).

Would disruption of endogenous KRAS alter follicle development, ovulation, or luteinization? The answer is

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probably yes, based on the potent, global effects of ERK1/2, a downstream target of RAS in granulosa cells. We would predict that a phenotype similar to the *Erk1/2* mutant mice (34) would be observed, assuming that other members of the RAS family do not compensate for KRAS. This is likely because the expression of KRAS^{G12D} alone in granulosa cells of small follicles silenced the endogenous *Kras* gene, leading to cell cycle arrest and the absence of ERK1/2 and AKT phosphorylation (19). One might not expect such a dramatic alteration of granulosa cell fate, if other RAS proteins maintained functional RAS activity. Moreover, recent studies have revealed marked functional differences among the highly related *Ras* family oncogenes. For example, nearly 50% of colon cancers harbor activating mutations in *Kras*, whereas *Nras* mutations occur in a smaller percentage (5%) (48). Conditional expression of *Kras*^{G12D} in the colonic epithelium stimulated hyperproliferation in a MEK-dependent manner. *Nras*^{G12D} did not alter the growth properties of the epithelium but was able to confer resistance to apoptosis (48). However, it is unclear whether the *Nras*^{G12D} mutant can induce the same changes in ovarian granulosa and epithelial cells, as the *Kras*^{G12D} does. Because *Hras* and *Nras* knockout mice are viable and fertile, we predict that these two *Ras* family members are unlikely to play an essential role in the ovary. Thus, it will be important to analyze the ovarian functions of endogenous *Kras* by using the *Loxp-Cre* system to selectively deplete *Kras* in granulosa cells. This *Kras* loss-of-function study will provide the valuable *in vivo* information complementary to the *Kras*^{G12D} gain-of-function studies we have conducted.

Does KRAS^{G12D} exert different effects in cells of other endocrine tissues than it does in epithelial cells and fibroblasts? The answer is probably yes, because KRAS^{G12D} causes premature cell cycle arrest as well as terminal differentiation of granulosa cells and because these cells do not express known markers of senescence, such as senescent-associated (SA)-β-galactosidase. Thus, these cells do not appear to be senescent (14, 15). Curiously, however, the terminal differentiation of granulosa cells to luteal cells (36, 41), stromal cells to decidual cells (37), and preadipocytes to adipocytes (38) appears to be mediated by potent cytokines and C/EBPβ, factors that also impact cell senescence and are markers of epithelial and fibroblast senescent cells (40, 80). A major difference is that RAS-induced senescent epithelial cells continue to produce copious amounts of cytokines (80) and continue to express the KRAS gene, whereas terminally differentiated granulosa cells do not.

Are the effects of *Kras*^{G12D} on cell cycle arrest dominant in granulosa cells but not OSE cells? The answer is probably yes, because GCTs arise in *Pten/Ctnnb1* mutant ovaries (75) but not in *Pten/Kras* mutant ovaries (19). These observations suggest that the cell cycle arrest power of KRAS^{G12D} is dominant to the potential tumorigenic (proliferative and antiapoptotic) effects of mutant *Pten*. By contrast, *Ctnnb1* and *Pten* mutations synergize to facilitate rampant proliferation of granulosa cells. Therefore, it is possible that *Kras*^{G12D} would also impede or reduce the tumorigenic effects of *Ctnnb1* or mutations of *Smad1*, *5*, or *8* or *inhibin* α in granulosa cells. These analyses remain to be done.

Furthermore, the ovarian cell-specific, *Kras*, *Pten*, and *Ctnnb1* mutant mice afford unique mouse models in which to analyze the molecular (mRNA and micro-RNA) and endocrine (hormone-dependent?) basis for tumor formation in OSE cells compared with the molecular and endocrine events that cause cell cycle arrest in granulosa cells. The *Pten/Kras* double-mutant mouse strain provides a potentially important model for OSE cancer because the onset and progression of OSE cell transformation in response to these oncogenes remains to be clearly defined. Multiple *Ras* downstream effectors such as the PI3K and ERK1/2 pathways are hyperactive in the OSE tumor cells (19). The contributions of each effector to the oncogenic transformation of OSE cells may lead to the discovery of new targets for cancer therapy concerning cancer.

Taken together, these studies of *Kras* in mammalian ovarian cells provide novel information relevant to how the RAS family of small G proteins may contribute to specific physiological and pathological processes within the ovary. The data clearly demonstrate the potent impact of endogenous RAS (KRAS) in regulating important physiological (follicle development and ovulation) events and the impact of mutant KRAS in dictating pathological (premature ovarian failure and OSE adenocarcinomas) outcomes. Hopefully, a better understanding of the molecular targets of RAS in specific ovarian cell types will build a bridge between normal reproductive biology and cancer biology in the ovary.

Acknowledgments

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References

1. **Bos JL** 1988 The ras gene family and human carcinogenesis. Mutat Res 195:255–271
2. **Barbacid M** 1987 Ras genes. Annu Rev Biochem 56:779–827
3. **Hall A** 1993 Ras-related proteins. Curr Opin Cell Biol 5:265–268
4. **Erdogan M, Pozzi A, Bhowmick N, Moses HL, Zent R** 2007 Signaling pathways regulating TC21-induced tumorigenesis. J Biol Chem 282:27713–27720
5. **Keduka E, Kaiho A, Hamada M, Watanabe-Takano H, Takano K, Ogasawara M, Satou Y, Satoh N, Endo T** 2009 M-Ras evolved independently of R-Ras and its neural function is conserved between mammalian and ascidian, which lacks classical Ras. Gene 429:49–58
6. **Lehto M, Mäyränpää MI, Pellinen T, Ihalmo P, Lehtonen S, Kovanen PT, Groop PH, Ivaska J, Olkkonen VM** 2008 The R-Ras interaction partner ORP3 regulates cell adhesion. J Cell Sci 121: 695–705
7. **Umanoff H, Edelmann W, Pellicer A, Kucherlapati R** 1995 The murine N-ras gene is not essential for growth and development. Proc Natl Acad Sci USA 92:1709–1713
8. **Ise K, Nakamura K, Nakao K, Shimizu S, Harada H, Ichise T, Miyoshi J, Gondo Y, Ishikawa T, Aiba A, Katsuki M** 2000 Targeted deletion of the H-ras gene decreases tumor formation in mouse skin carcinogenesis. Oncogene 19:2951–2956
9. **Koera K, Nakamura K, Nakao K, Miyoshi J, Toyoshima K, Hatta T, Otani H, Aiba A, Katsuki M** 1997 K-ras is essential for the development of the mouse embryo. Oncogene 15:1151–1159
10. **Campbell PM, Groehler AL, Lee KM, Ouellette MM, Khazak V, Der CJ** 2007 K-Ras promotes growth transformation and invasion of immortalized human pancreatic cells by Raf and phosphatidylinositol 3-kinase signaling. Cancer Res 67:2098–2106
11. **Céspedes MV, Sancho FJ, Guerrero S, Parreño M, Casanova I, Pavón MA, Marcuello E, Trias M, Cascante M, Capellà G, Mangues R** 2006 K-ras Asp12 mutant neither interacts with Raf, nor signals through Erk and is less tumorigenic than K-ras Val12. Carcinogenesis 27:2190–2200
12. **Gupta S, Ramjaun AR, Haiko P, Wang Y, Warne PH, Nicke B, Nye E, Stamp G, Alitalo K, Downward J** 2007 Binding of Ras to phosphoinositide 3-kinase p110α is required for Ras-driven tumorigenesis in mice. Cell 129:957–968
13. **Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW** 1997 Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88:593–602
14. **Courtois-Cox S, Genther Williams SM, Reczek EE, Johnson BW, McGillicuddy LT, Johannessen CM, Hollstein PE, MacCollin M, Cichowski K** 2006 A negative feedback signaling network underlies oncogene-induced senescence. Cancer Cell 10:459–472
15. **Courtois-Cox S, Jones SL, Cichowski K** 2008 Many roads lead to oncogene-induced senescence. Oncogene 27:2801–2809
16. **Wayne CM, Fan HY, Cheng X, Richards JS** 2007 Follicle-stimulating hormone induces multiple signaling cascades: evidence that activation of Rous sarcoma oncogene, RAS, and the epidermal growth factor receptor are critical for granulosa cell differentiation. Mol Endocrinol 21:1940–1957
17. **Needle E, Piparo K, Cole D, Worrall C, Whitehead I, Mahon G, Goldsmith LT** 2007 Protein kinase A-independent cAMP stimulation of progesterone in a luteal cell model is tyrosine kinase dependent but phosphatidylinositol-3-kinase and mitogen-activated protein kinase independent. Biol Reprod 77:147–155
18. **Fan HY, Shimada M, Liu Z, Cahill N, Noma N, Wu Y, Gossen J, Richards JS** 2008 Selective expression of KrasG12D in granulosa cells of the mouse ovary causes defects in follicle development and ovulation. Development 135:2127–2137
19. **Fan HY, Liu Z, Paquet M, Wang J, Lydon JP, DeMeyo F, Richards JS** 2009 Cell type-specific targeted mutations of Kras and Pten document proliferation arrest in granulosa cells versus oncogenic insult to ovarian surface epithelial cells. Cancer Res 69:6463–6472
20. **Hunzicker-Dunn M, Maizels ET** 2006 FSH signaling pathways in immature granulosa cells that regulate target gene expression: branching out from protein kinase A. Cell Signal 18:1351–1359
21. **Richards JS** 1994 Hormonal control of gene expression in the ovary. Endocr Rev 15:725–751
22. **Matzuk MM, Burns KH, Viveiros MM, Eppig JJ** 2002 Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 296:2178–2180
23. **Richards JS** 2001 New signaling pathways for hormones and cyclic adenosine 3′,5′-monophosphate action in endocrine cells. Mol Endocrinol 15:209–218
24. **Gonzalez-Robayna IJ, Falender AE, Ochsner S, Firestone GL, Richards JS** 2000 Follicle-stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-induced kinase (Sgk): evidence for A kinase-independent signaling by FSH in granulosa cells. Mol Endocrinol 14:1283–1300
25. **Charest PG, Oligny-Longpré G, Bonin H, Azzi M, Bouvier M** 2007 The V2 vasopressin receptor stimulates ERK1/2 activity independently of heterotrimeric G protein signalling. Cell Signal 19:32–41
26. **Wang Q, Lu R, Zhao J, Limbird LE** 2006 Arrestin serves as a molecular switch, linking endogenous α2-adrenergic receptor to SRC-dependent, but not SRC-independent, ERK activation. J Biol Chem 281:25948–25955
27. **Miller WE, Lefkowitz RJ** 2001 Expanding roles for β-arrestins as scaffolds and adapters in GPCR signaling and trafficking. Curr Opin Cell Biol 13:139–145
28. **Shiraishi K, Ascoli M** 2006 Activation of the lutropin/choriogonadotropin receptor in MA-10 cells stimulates tyrosine kinase cascades that activate ras and the extracellular signal regulated kinases (ERK1/2). Endocrinology 147:3419–3427
29. **Park JY, Su YQ, Ariga M, Law E, Jin SL, Conti M** 2004 EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science 303:682–684
30. **Hernandez-Gonzalez I, Gonzalez-Robayna I, Shimada M, Wayne CM, Ochsner SA, White L, Richards JS** 2006 Gene expression profiles of cumulus cell oocyte complexes during ovulation reveal cumulus cells express neuronal and immune-related genes: does this expand their role in the ovulation process? Mol Endocrinol 20: 1300–1321
31. **Shimada M, Hernandez-Gonzalez I, Gonzalez-Robayna I, Richards JS** 2006 Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: key roles for prostaglandin synthase 2 and progesterone receptor. Mol Endocrinol 20:1352–1365
32. **Hsieh M, Lee D, Panigone S, Horner K, Chen R, Theologis A, Lee DC, Threadgill DW, Conti M** 2007 Luteinizing hormone-dependent activation of the epidermal growth factor network is essential for ovulation. Mol Cell Biol 27:1914–1924
33. **Espey LL, Richards JS** 2002 Temporal and spatial patterns of ovarian gene transcription following an ovulatory dose of gonadotropin in the rat. Biol Reprod 67:1662–1670
34. **Fan HY, Liu Z, Shimada M, Sterneck E, Johnson PF, Hedrick SM, Richards JS** 2009 MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. Science 324:938–941
35. **Panigone S, Hsieh M, Fu M, Persani L, Conti M** 2008 Luteinizing hormone signaling in preovulatory follicles involves early activation of the epidermal growth factor receptor pathway. Mol Endocrinol 22:924–936
36. **Sterneck E, Tessarollo L, Johnson PF** 1997 An essential role for C/EBPβ in female reproduction. Genes Dev 11:2153–2162
37. **Mantena SR, Kannan A, Cheon YP, Li Q, Johnson PF, Bagchi IC, Bagchi MK** 2006 C/EBPβ is a critical mediator of steroid hormone-regulated cell proliferation and differentiation in the uterine epithelium and stroma. Proc Natl Acad Sci USA 103:1870–1875
38. **Schaffler A, Scholmerich J, Salzberger B** 2007 Adipose tissue as an immunological organ: Toll-like receptors, C1q/TNFs and CTRPs. Trends Immunol 28:393–399

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39. **Sebastian T, Malik R, Thomas S, Sage J, Johnson PF** 2005 C/EBPβ cooperates with RB:E2F to implement Ras(V12)-induced cellular senescence. EMBO J 24:3301–3312
40. **Kuilman T, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ, Aarden LA, Mooi WJ, Peeper DS** 2008 Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. Cell 133:1019–1031
41. **Liu Z, de Matos DG, Fan HY, Shimada M, Palmer S, Richards JS** 2009 IL6: an autocrine regulator of the mouse cumulus cell-oocyte complex expansion process. Endocrinology 150:3360–3368
42. **Duggavathi R, Volle DH, Mataki C, Antal MC, Messaddeq N, Auwerx J, Murphy BD, Schoonjans K** 2008 Liver receptor homolog 1 is essential for ovulation. Genes Dev 22:1871–1876
43. **Jeyasuria P, Ikeda Y, Jamin SP, Zhao L, De Rooij DG, Themmen AP, Behringer RR, Parker KL** 2004 Cell-specific knockout of steroidogenic factor 1 reveals its essential roles in gonadal function. Mol Endocrinol 18:1610–1619
44. **Lee YK, Choi YH, Chua S, Park YJ, Moore DD** 2006 Phosphorylation of the hinge domain of the nuclear hormone receptor LRH-1 stimulates transactivation. J Biol Chem 281:7850–7855
45. **Su YQ, Wigglesworth K, Pendola FL, O'Brien MJ, Eppig JJ** 2002 Mitogen-activated protein kinase activity in cumulus cells is essential for gonadotropin-induced oocyte meiotic resumption and cumulus expansion in the mouse. Endocrinology 143:2221–2232
46. **Sarkisian CJ, Keister BA, Stairs DB, Boxer RB, Moody SE, Chodosh LA** 2007 Dose-dependent oncogene-induced senescence in vivo and its evasion during mammary tumorigenesis. Nat Cell Biol 9:493–505
47. **Johnson L, Mercer K, Greenbaum D, Bronson RT, Crowley D, Tuveson DA, Jacks T** 2001 Somatic activation of the K-ras oncogene causes early onset lung cancer in mice. Nature 410:1111–1116
48. **Haigis KM, Kendall KR, Wang Y, Cheung A, Haigis MC, Glickman JN, Niwa-Kawakita M, Sweet-Cordero A, Sebolt-Leopold J, Shannon KM, Settleman J, Giovannini M, Jacks T** 2008 Differential effects of oncogenic K-Ras and N-Ras on proliferation, differentiation and tumor progression in the colon. Nat Genet 40:600–608
49. **Tuveson DA, Shaw AT, Willis NA, Silver DP, Jackson EL, Chang S, Mercer KL, Grochow R, Hock H, Crowley D, Hingorani SR, Zaks T, King C, Jacobetz MA, Wang L, Bronson RT, Orkin SH, DePinho RA, Jacks T** 2004 Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. Cancer Cell 5:375–387
50. **Shaw AT, Meissner A, Dowdle JA, Crowley D, Magendantz M, Ouyang C, Parisi T, Rajagopal J, Blank LJ, Bronson RT, Stone JR, Tuveson DA, Jaenisch R, Jacks T** 2007 Sprouty-2 regulates oncogenic K-ras in lung development and tumorigenesis. Genes Dev 21:694–707
51. **Orsulic S, Li Y, Soslow RA, Vitale-Cross LA, Gutkind JS, Varmus HE** 2002 Induction of ovarian cancer by defined multiple genetic changes in a mouse model system. Cancer Cell 1:53–62
52. **Connolly DC, Bao R, Nikitin AY, Stephens KC, Poole TW, Hua X, Harris SS, Vanderhyden BC, Hamilton TC** 2003 Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer. Cancer Res 63:1389–1397
53. **Dinulescu DM, Ince TA, Quade BJ, Shafer SA, Crowley D, Jacks T** 2005 Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer. Nat Med 11:63–70
54. **Wu R, Hendrix-Lucas N, Kuick R, Zhai Y, Schwartz DR, Akyol A, Hanash S, Misek DE, Katabuchi H, Williams BO, Fearon ER, Cho KR** 2007 Mouse model of human ovarian endometrioid adenocarcinoma based on somatic defects in the Wnt/β-catenin and PI3K/Pten signaling pathways. Cancer Cell 11:321–333
55. **Pieretti-Vanmarcke R, Donahoe PK, Pearsall LA, Dinulescu DM, Connolly DC, Halpern EF, Seiden MV, MacLaughlin DT** 2006 Mullerian Inhibiting Substance enhances subclinical doses of chemotherapeutic agents to inhibit human and mouse ovarian cancer. Proc Natl Acad Sci USA 103:17426–17431
56. **Jorgez CJ, Klysik M, Jamin SP, Behringer RR, Matzuk MM** 2004 Granulosa cell-specific inactivation of follistatin causes female fertility defects. Mol Endocrinol 18:953–967
57. **Hernandez Gifford JA, Hunzicker-Dunn ME, Nilson JH** 2009 Conditional deletion of β-catenin mediated by Amhr2cre in mice causes female infertility. Biol Reprod 80:1282–1292
58. **Fan HY, Liu Z, Cahill N, Richards JS** 2008 Targeted disruption of Pten in ovarian granulosa cells enhances ovulation and extends the life span of luteal cells. Mol Endocrinol 22:2128–2140
59. **Soyal SM, Mukherjee A, Lee KY, Li J, Li H, DeMayo FJ, Lydon JP** 2005 Cre-mediated recombination in cell lineages that express the progesterone receptor. Genesis 41:58–66
60. **Krimpenfort P, Ijpenberg A, Song JY, van der Valk M, Nawijn M, Zevenhoven J, Berns A** 2007 p15Ink4b is a critical tumour suppressor in the absence of p16Ink4a. Nature 448:943–946
61. **Sebastian T, Johnson PF** 2006 Stop and go: anti-proliferative and mitogenic functions of the transcription factor C/EBPβ. Cell Cycle 5:953–957
62. **Ancrile B, Lim KH, Counter CM** 2007 Oncogenic Ras-induced secretion of IL6 is required for tumorigenesis. Genes Dev 21:1714–1719
63. **Kurman RJ, Shih IeM** 2008 Pathogenesis of ovarian cancer: lessons from morphology and molecular biology and their clinical implications. Int J Gynecol Pathol 27:151–160
64. **Vanderhyden BC, Shaw TJ, Ethier JF** 2003 Animal models of ovarian cancer. Reprod Biol Endocrinol 1:67
65. **Shah SP, Köbel M, Senz J, Morin RD, Clarke BA, Wiegand KC, Leung G, Zayed A, Mehl E, Kalloger SE, Sun M, Giuliany R, Yorida E, Jones S, Varhol R, Swenerton KD, Miller D, Clement PB, Crane C, Madore J, Provencher D, Leung P, DeFazio A, Khattra J, Turashvili G, Zhao Y, Zeng T, Glover JN, Vanderhyden B, Zhao C, Parkinson CA, Jimenez-Linan M, Bowtell DD, Mes-Masson AM, Brenton JD, Aparicio SA, Boyd N, Hirst M, Gilks CB, Marra M, Huntsman DG** 2009 Mutation of FOXL2 in granulosa-cell tumors of the ovary. N Engl J Med 360:2719–2729
66. **Matzuk MM, Finegold MJ, Su JG, Hsueh AJ, Bradley A** 1992 α-Inhibin is a tumour-suppressor gene with gonadal specificity in mice. Nature 360:313–319
67. **Li Q, Graff JM, O'Connor AE, Loveland KL, Matzuk MM** 2007 SMAD3 regulates gonadal tumorigenesis. Mol Endocrinol 21: 2472–2486
68. **Pangas SA, Li X, Umans L, Zwijsen A, Huylebroeck D, Gutierrez C, Wang D, Martin JF, Jamin SP, Behringer RR, Robertson EJ, Matzuk MM** 2008 Conditional deletion of Smad1 and Smad5 in somatic cells of male and female gonads leads to metastatic tumor development in mice. Mol Cell Biol 28:248–257
69. **Boerboom D, Paquet M, Hsieh M, Liu J, Jamin SP, Behringer RR, Sirois J, Taketo MM, Richards JS** 2005 Misregulated Wnt/β-catenin signaling leads to ovarian granulosa cell tumor development. Cancer Res 65:9206–9215
70. **Boerboom D, White LD, Dalle S, Courty J, Richards JS** 2006 Dominant-stable β-catenin expression causes cell fate alterations and Wnt signaling antagonist expression in a murine granulosa cell tumor model. Cancer Res 66:1964–1973
71. **Cantley LC, Neel BG** 1999 New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci USA 96:4240–4245
72. **Li G, Robinson GW, Lesche R, Martinez-Diaz H, Jiang Z, Rozengurt N, Wagner KU, Wu DC, Lane TF, Liu X, Hennighausen L, Wu H** 2002 Conditional loss of PTEN leads to precocious development and neoplasia in the mammary gland. Development 129:4159–4170
73. **Richards JS, Sharma SC, Falender AE, Lo YH** 2002 Expression of FKHR, FKHRL1, and AFX genes in the rodent ovary: evidence for

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regulation by IGF-I, estrogen, and the gonadotropins. Mol Endocrinol 16:580–599

74. **Shi F, LaPolt PS** 2003 Relationship between FoxO1 protein levels and follicular development, atresia, and luteinization in the rat ovary. J Endocrinol 179:195–203
75. **Laguë MN, Paquet M, Fan HY, Kaartinen MJ, Chu S, Jamin SP, Behringer RR, Fuller PJ, Mitchell A, Doré M, Huneault LM, Richards JS, Boerboom D** 2008 Synergistic effects of Pten loss and WNT/CTNNB1 signaling pathway activation in ovarian granulosa cell tumor development and progression. Carcinogenesis 29:2062–2072
76. **Wang Y, Cheon DJ, Lu Z, Cunningham SL, Chen CM, Luo RZ, Xing D, Orsulic S, Bast Jr RC, Behringer RR** 2008 MUC16 expression during embryogenesis, in adult tissues, and ovarian cancer in the mouse. Differentiation 76:1081–1092
77. **Kumar TR, Low MJ, Matzuk MM** 1998 Genetic rescue of follicle-stimulating hormone β-deficient mice. Endocrinology 139:3289–3295
78. **Risma KA, Clay CM, Nett TM, Wagner T, Yun J, Nilson JH** 1995 Targeted overexpression of luteinizing hormone in transgenic mice leads to infertility, polycystic ovaries, and ovarian tumors. Proc Natl Acad Sci USA 92:1322–1326
79. **Bliss SP, Miller A, Navratil AM, Xie J, McDonough SP, Fisher PJ, Landreth GE, Roberson MS** 2009 ERK signaling in the pituitary is required for female but not male fertility. Mol Endocrinol 23:1092–1101
80. **Coppé JP, Patil CK, Rodier F, Sun Y, Muñoz DP, Goldstein J, Nelson PS, Desprez PY, Campisi J** 2008 Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol 6:2853–2868

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