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Cell Type-Specific Targeted Mutations of *Kras* and *Pten* Document Proliferation Arrest in Granulosa Cells Versus Oncogenic Insult to Ovarian Surface Epithelial Cells

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Abstract

The small G-protein KRAS is crucial for mediating gonadotropin-induced events associated with ovulation. However, constitutive expression of $Kras^{G12D}$ in granulosa cells disrupted normal follicle development leading to the persistence of abnormal follicle-like structures containing non-mitotic cells. To determine what factors mediate this potent effect of $Kras^{G12D}$, gene profiling analyses were done. We also analyzed $Kras^{G12D}$*;Cyp19-Cre* and $Kras^{G12D}$*;Pgr-Cre* mutant mouse models that express Cre prior to or after the initiation of granulosa cell differentiation, respectively. $Kras^{G12D}$ induced cell cycle arrest in granulosa cells of the $Kras^{G12D}$*;Cyp19-Cre* mice but not in the $Kras^{G12D}$*;Pgr-Cre* mice, documenting the cell context specific effect of $Kras^{G12D}$. Expression of $Kras^{G12D}$ silenced the *Kras* gene, reduced cell cycle activator genes and impaired expression of granulosa cell and oocyte specific genes. Conversely, levels of PTEN and phosphorylated p38MAPK increased markedly in the mutant granulosa cells. Because disrupting *Pten* in granulosa cells leads to increased proliferation and survival, *Pten* was disrupted in the $Kras^{G12D}$ mutant mice. The *Pten/Kras* mutant mice were infertile but lacked granulosa cell tumors. By contrast, the $Pten^{fl/fl}$*;*$Kras^{G12D}$*;Amhr2-Cre* mice developed aggressive ovarian surface epithelial (OSE) cell tumors that did not occur in the $Pten^{fl/fl}$*;*$Kras^{G12D}$*;Cyp19-Cre* or $Pten^{fl/fl}$*;*$Kras^{G12D}$*;Pgr-Cre* mouse strains. These data document unequivocally that *Amhr2-Cre* is expressed in and mediates allelic recombination of oncogenic genes in OSE cells. That $Kras^{G12D}$*/Pten* mutant granulosa cells do not transform but rather undergo cell cycle arrest indicates that they resist the oncogenic insults of *Kras/Pten* by robust self-protecting mechanisms that silence the *Kras* gene and elevate PTEN and phospho-p38MAPK.

Keywords

ovary; granulosa cell; *Kras*; *Pten*; oncogenes; ovarian cancer; follicle development

Introduction

Ovarian cancer represents a major threat to women's health because our knowledge of the underlying causes of this complex and heterogeneous disease remains ill defined. Ovarian carcinomas of the ovarian surface epithelium (OSE) are the most aggressive and prevalent,

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accounting for ~90% of all human epithelial ovarian cancers and have been classified based on histological criteria as serous, endometrioid, clear cell and mucinous. By contrast, only 5% of human ovarian cancers are derived from granulosa cells (1-3). This seems unexpected because granulosa cells are the most abundant proliferative cell type in the ovary. It is not known if the low incidence of granulosa cell tumors (GCTs) is due to the strong resistance of granulosa cells to oncogenic stimuli, the rare occurrence of oncogenic mutations in this population of ovarian cells or to the natural fate of granulosa cells that are removed from the proliferative stage either by apoptosis or the natural progression to terminally differentiated non-dividing luteal cells.

The proliferation and differentiation of granulosa cells in developing follicles are regulated by the pituitary gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) and their G-protein coupled receptors as well as by ovarian derived growth regulatory factors (4,5). Recent studies document that in addition to the canonical cAMP/protein kinase A pathway, FSH and LH activate several other signaling cascades, such as the RAF1/MEK/MAPK3/1 (also known as ERK1/2) pathway and the PI3K/AKT pathway (6-9) via activation of the small G-protein RAS and the tyrosine kinase SRC, respectively (7). Conditional expression of an oncogenic $Kras^{G12D}$ mutation in granulosa cells impacts both the ERK1/2 and PI3K pathways and leads to defects in follicle development and ovulation (10). Surprisingly, whereas $Kras^{G12D}$ mutations induce tumors in lung (11), mammary gland (12) and uterus (Wang J and DeMayo F, unpublished results), it does not stimulate the oncogenic transformation of granulosa cells. Rather, the $Kras^{G12D}$ expressing granulosa cells undergo cell cycle arrest, are non-mitotic based on the absence of either BrdU labeling or phospho-histone H3 immuno-staining, are non-apoptotic based on the lack of TUNEL positive cells or cells exhibiting cleaved caspase 3 immuno-staining and fail to differentiate [10]. The abnormal follicle-like structures are devoid of oocytes and persist in the mutant ovaries for prolonged periods of time (10).

The PI3K pathway is well recognized as essential for cell survival and proliferation; its hyper-activation is associated with oncogenesis in multiple tissue and cell types (13,14). The phosphatidylinositol phosphate phosphatase PTEN (phosphatase tensin homolog) negatively regulates the activity of PI3K pathway and therefore functions as a potent tumor repressor. Although mutations of the *Pten* locus can lead to tumor formation in many but not all tissues (15,16), the disruption of the *Pten* gene causes embryonic lethality in mice, indicating that it is also essential for normal development (17). Selective depletion of *Pten* in mouse granulosa cells leads to hyper-activation of the PI3K pathway, increased follicle growth and ovulation, and the prolonged life-span of corpora lutea (18) but rarely induces GCTs (19).

In this study, we sought to determine the cell specific and combinatorial effects of expressing $Kras^{G12D}$ and mutant *Pten* in mouse ovarian cells. Using six different mouse models we document that the $Kras^{G12D}$ mutation in granulosa cells induces cell cycle arrest in a developmental stage-dependent manner and that mice with double mutations of *Pten/Kras* selectively in granulosa cells were infertile but showed no GCTs. By contrast, in the $Pten^{fl/fl}$;$Kras^{G12D}$;*Amhr2Cre* mice where double mutations are expressed in granuloss cells and the OSE (OSE) cells, massive ovarian serous papillary adenocarcinomas develop at an early age. These results indicate that granulosa cells are highly resistant to these oncogenic factors that transform OSE cells and therefore possess robust self-protecting mechanisms that arrest cell cycle progression.

Materials and Methods

Animals

LSL-Kras^{G12D};Amhr2-Cre, LSL-Kras^{G12D};Cyp19-Cre, LSL-Kras^{G12D};Pgr-Cre, Pten$^{fl/fl}$;LSL-Kras^{G12D};Amhr2-Cre, Pten$^{fl/fl}$;LSL-Kras^{G12D};Cyp19-Cre and *Pten$^{fl/fl}$;LSL-Kras^{G12D};Pgr-Cre* mice were derived from previously described *Amhr2-Cre, Cyp19-Cre, Pgr-Cre, Pten$^{fl/fl}$* and *LSL-K-ras^{G12D}* parental strains, and genotyping was done by PCR as described (10,20-23). Histological images and the efficient recombination of the mutant alleles was documented for each genotype (Suppl. Fig. 2G). Animals were housed under a 16-h light/8-h dark schedule in the Center for Comparative Medicine at Baylor College of Medicine and provided food and water *ad libitum*. Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee at Baylor College of Medicine.

Immature 21 day-old female mice were injected intraperitoneally (ip) with 4IU of eCG (equine chorionic gonadotropin; Calbiochem, San Diego, CA) to stimulate follicular growth followed 48h later with 5IU hCG (human chorionic gonadotropin, American Pharmaceutical Partners, Schaumburg, IL) to stimulate ovulation and luteinization. Ovulated oocytes were collected from oviducts 16h after hCG injection. Development of ovarian tumors was checked in mice at the age of 1 month, 3 months, and 6 months.

Histology and immunohistochemistry

Ovaries were collected and fixed in 4% paraformaldehyde (PFA) and embedded in paraffin and processed by routine procdures (10). Immunohistochemistry was done as previously described [10] using antibodies against cytokeratin 8 (Abcam, Cambridge, MA), PTEN (Cell Signaling, Danvers, MA) or PCNA (Cell Signaling, Danvers, MA). Sections were counterstained with hematoxylin, dehydrated, and mounted.

Immunofluorescence

Ovaries were fixed in 4% PFA, embedded in O.C.T. compound (Sakura Finetek USA Inc.) and stored at -70°C before the preparation of 7μM sections and immunostaining (10). Antibodies for WT1, KRAS, Cyclin A and E2F1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA. All the other antibodies were purchased from Cell Signaling. The MUC16 antibody was gifted from Dr. Robert Bast, Jr.

BrdU incorporation assay

BrdU and a monoclonal antibody against BrdU (Sigma Chemical Company, St. Louis, MO) were used as previously described (10).

Hormone assays

Radioimmunoassays for FSH and estradiol were performed by the Ligand Assay and Analysis Core, SCCPIR (University of Virginia), as previously described (24).

Microarray analyses

Total ovarian RNA was isolated from 3-month-old *Kras^{G12D};Amhr2-Cre* mice and immature 26 day old control mice using the RNeasy Mini kit (Qiagen Sciences, Germantown, MD). At 3 months of age, 100% of the *Kras^{G12D};Amhr2-Cre* ovaries contain an abundance of abnormal follicles but not corpora lutea whereas the immature mouse ovaries contain predominantly normal follicles. Thus, this was deemed the most appropriate comparison for detecting granulosa cell genes. To avoid ovarian related differences in gene expression that could arise by comparing single ovaries, RNA samples from 3 animals per genotype were pooled before

microarray probe synthesis. Wild type and *Kras^{G12D};Amhr2-Cre* riboprobes were then hybridized to Mouse 430.2 microarray chips (Affymetrix, Santa Clara, CA). All steps of RNA quality control, probe synthesis, hybridization, washing, array scanning, and statistical analyses were done by the Microarray Core Facility of the Baylor College of Medicine (Houston, TX) as previously described (25).

RNA isolation and real-time PCR

For real-time RT-PCR analyses additional total ovarian RNA was also isolated from ovaries of control (26 day and 3 month old) mice, *Kras^{G12D};Amhr2-Cre* (3month old) mice and *Pten$^{fl/fl}$;Kras^{G12D};Amhr2-Cre* (3 month old) mice. Reverse transcription was done using the SuperScript One-Step RT-PCR system with Platinum Taq kit (Invitrogen, Carlsbad, CA). The real-time PCR was performed using the Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia). The PCR reaction included 5 μl of SYBR Green JumpStart Taq Mix (Sigma), 4.0μl first strand cDNA product described above (1:100 dilution), and 0.5μM of forward and reverse primers. Relative levels of gene expression were calculated using Rotor-Gene 6.0 software and normalized to beta-actin.

Western Blot Analysis

Tissues and cells were lysed with RIPA buffer containing complete protease inhibitors (Roche) (10). Western blots were performed utilizing 30μg of lysate protein, incubated with antibodies specific for: FOXO1, FOXO3, PTEN, AKT, cyclin D2 and phospho-mTOR, -GSK3β, -PDK1, -ERK1/2, -AKT, (all from Cell Signaling Technology, 1:1000 dilution), cyclin A (Santa Cruz Biotechnology), E2F1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical analyses

The data for real-time RT-PCR assays, breeding experiments, hormone levels and super-ovulation tests are represented as means ± SD. Data were analyzed by using GraphPad Prism Programs (ANOVA or t test; GraphPad Prism, San Diego, CA) to determine significance. Values were considered significantly different if $P \leq 0.05$ or $P \leq 0.01$.

Results

Kras^{G12D} induction of cell cycle arrest in ovarian granulosa cells is stage-dependent

The *Kras^{G12D};Amhr2-Cre* mutant mice contain abnormal follicle-like structures consisting of non-mitotic and non-apoptotic granulosa cells that persist for prolonged periods of time (10). As shown herein, the ovaries of *Kras^{G12D};Amhr2-Cre* mice progressively increase in size as the mice age and this is associated with the accumulation of the small follicle-derived ovarian lesions (Fig. 1A, for each time point or genotype, ovaries from at least 4 animals were sectioned, and ovarian lesions were counted in 6 sections of each ovary). To determine if the formation of these abnormal follicle-like structures was dependent on the stage of granulosa cell differentiation, we generated three *Kras^{G12D}* mutant mouse models using mouse strains (*Amhr2-Cre*, *Cyp19-Cre* and *Pgr-Cre*) in which *Cre* is driven by promoters expressed at different stages of follicular development *in vivo*. In the *Amhr2-Cre* and *Cyp19-Cre* females, Cre-mediated allelic recombination occurs primarily in proliferating granulosa cells of primary follicles and early antral follicles, respectively. Because the expression of the endogenous progesterone receptor (*Pgr*) gene is induced only in granulosa cells of preovulatory follicles exposed to the LH surge, Cre-mediated allelic recombination in the *Pgr-Cre* mice is restricted to terminally differentiating granulosa cells undergoing luteinization (Fig. 1B) (23,26). When the ovaries of the three mutant mouse models were compared at 6 months of age, the ovaries of *Kras^{G12D};Amhr2-Cre* females contained 2-fold more abnormal follicle-like structures than those of the *Kras^{G12D};Cyp19-Cre* mice (Fig. 1A). Strikingly, ovaries of the *Kras^{G12D};Pgr-*

Cre females contained no abnormal follicle-like structures and were normal (Fig. 1B), indicating that the *Kras*^{G12D} mutation initiates inappropriate cell cycle arrest only at an early stage of follicle development when granulosa cells are proliferating.

Kras^{G12D} alters cell cycle and differentiation related gene expression profiles in granulosa cells

Gene expression profiling (Suppl. Table 1) and real-time RT-PCR (Fig. 3B) using total RNA from ovaries of *Kras*^{G12D}*;Amhr2-Cre* mice documented that genes regulating cell cycle progression (27), especially cyclins and members of the E2F transcription factor family, were down-regulated whereas the genes, *Pten* and *Zbtb16* (also known as *Plzf*) that have been reported to repress cell cycle progression (28), were up-regulated (Suppl. Table 1, Fig. 3B). Genes that control granulosa cell differentiation [FSH receptor (*Fshr*), activin/inhibin (*Inhba*/*Inhbb/Inha*) and aromatase (*Cyp19*)] and genes encoding key transcription factors *Fos*, *Foxo1* and *Nr5a2* were down-regulated in the *Kras*^{G12D} mutant cells (Suppl. Table 1). By contrast, expression levels of the LH receptor (*Lhcgr*) and the cholesterol side chain cleavage cytochrome P450 enzyme (*Cyp11a1*) increased and are likely associated with the hypertrophy of interstitial cells in the *Kras* mutant ovaries.

Kras^{G12D} mediated cell cycle arrest in ovarian granulosa cells is associated with elevated PTEN and phosphorylated p38MAPK and reduced expression of KRAS

KRAS is highly expressed in granulosa cells of normal growing follicles (Fig. 2A WT), but was undetectable in the non-mitotic granulosa cells within the abnormal follicles (Fig. 2A: *Kras;Amhr2-Cre*). Moreover, the response of granulosa cells to KRAS^{G12D} is dependent on the level of the mutant the KRAS expressed in these cells (Suppl. Fig. 1A-C) supporting the evidence that p15^{INK4B}, p16^{INK4A} and TRP53 are unlikely to mediate the effects of KRAS^{G12D} in granulosa cells of the *Kras;Amhr2-Cre* mice *in vivo*.

Because the microarray results indicated that *Pten* mRNA levels increased in the *Kras* mutant ovaries, the cell specific expression of PTEN protein was analyzed and shown to be low in granulosa cells of the growing follicles present in ovaries of control mice compared to interstitial cells and corpora lutea (Fig. 2B, WT) (18). In marked contrast, PTEN was dramatically elevated in cells within the follicle lesions present in ovaries of the *Kras* mutant mice (Fig. 2B, *Kras;Amhr2-Cre*;Supp. Fig. 4). Elevated PTEN in the mutant granulosa cells was associated with negligible immuno-staining for phospho-AKT compared to granulosa cells in normal growing follicles (Fig. 2C). In contrast to the suppression of the KRAS-ERK1/2 and AKT pathways in the *Kras*^{G12D} mutant granulosa cells, elevated levels of phospho-p38MAPK (pMAPK14) were observed in these cells (Fig. 2C). Although phospho-p38MAPK is commonly associated with oncogenic-induced cell cycle arrest and senescence in fibroblasts and epithelial cells (29,30), the *Kras*^{G12D} mutant granulosa cells did not express (SA)-beta-galactosidase (31) or the secretory factor, IL6 (32)(data not shown). Thus, the complete silencing of the *Kras* gene (Fig. 2A) and down-regulation of the cell cycle regulatory molecules (Suppl. Table 1) combined with up-regulation of PTEN and phospho-p38MAPK appear to characterize the phenotype in the cycle arrested granulosa cells.

Double mutations of *Pten* and *Kras* in granulosa cells do not lead to tumors but do disrupt ovarian function and fertility leading to premature ovarian failure

Because disrupting *Pten* alone in ovarian granulosa cells in the *Pten*$^{fl/fl}$*;Cyp19-Cre* mice increases fertility and leads to the extended survival and life-span of granulosa/luteal cells (18) and only rarely leads to GCTs in the *Pten*$^{fl/fl}$*;Amhr2-Cre* mice (19), we next sought to determine if disrupting *Pten* in the *Kras* mutant genotypes would lead to tumor formation. Contrary to our hypothesis, double mutations of *Pten* and *Kras* in granulosa cells of the *Kras*^{G12D}*;Amhr2-Cre* and *Kras*^{G12D}*;Cyp19-Cre* mice failed to cause the development of

GCTs but altered follicular growth, prevented ovulation and rendered the mice infertile (Fig. 3A), a phenotype more severe than that of the *Kras^{G12D};Amhr2-Cre* and *Kras^{G12D};Cyp19-Cre* females (10). These results indicate that the *Kras* mutation plays a dominant role in granulosa cells and that the conditional knockout of *Pten* strengthened the effect of *Kras^{G12D}*.

Histopathological examination of ovarian morphology indicated that the *Pten$^{fl/fl}$;Kras^{G12D};Cyp19-Cre* double mutant mice showed signs of premature ovarian failure. Ovaries from females older than 3 months had reduced numbers of developing follicles (Fig. 3A), high levels of serum FSH and low levels of serum estradiol (Fig. 3A). Furthermore, granulosa cell marker genes and oocyte-specific genes were dramatically decreased in *Kras* mutant ovaries (Suppl. Table 1, Fig. 3B).

Western blot and immunofluorescent analyses show that cyclin D2, cyclin A, and E2F1 are highly expressed in granulosa cells of wild type mice but are markedly reduced in ovaries of 3 month old *Pten$^{fl/fl}$;Kras^{G12D};Cyp19-Cre* mice (Fig. 3C-D and data not shown). Immuno-staining of cyclin A and E2F1 was negligible in granulosa cells present in the abnormal follicle-like structures compared to the normal-looking follicles in the 1-month-old *Pten$^{fl/fl}$;Kras^{G12D};Cyp19-Cre* ovaries (Fig. 3D).

Double mutations of *Pten* and *Kras* in the *Pten$^{fl/fl}$;Kras^{G12D};Amhr2-Cre* mice but not the *Pten$^{fl/fl}$;Kras^{G12D};Cyp19-Cre* mice cause OSE cancer

Although GCTs did not develop in response to double mutations in *Kras^{G12D}* and *Pten*, the ovarian surface epithelium (OSE) was affected selectively when recombination of both genes was driven by *Amhr2-Cre*. The *Pten$^{fl/fl}$;Kras^{G12D};Amhr2-Cre* mice developed aggressive OSE tumors as early as 1 month of age (Fig. 4A/B: *Pten;Kras;A-Cre*) whereas the *Pten$^{fl/fl}$;Kras^{G12D};Cyp19-Cre* females did not. (Fig. 4A/B: *Pten;Kras;C-Cre*; Suppl. Fig. 2). The ovarian tumors present in the *Pten$^{fl/fl}$;Kras^{G12D};Amhr2-Cre* mice have been classified as low grade serous papillary cystadenocarcinomas by histological criteria (Fig. 4; Suppl. Fig. 2&3) and by immuno-staining of ovarian sections with the epithelial cell marker cytokeratin 8 (CK8) (Fig. 4C), the OSE cell marker MUC 16 and the serous adenocarcinoma cell marker WT-1 (Suppl. Fig. 3A-C). The development of OSE cell tumors in the *Amhr2-Cre* mouse strain but not the *Cyp19-Cre* strain is caused by the expression of *Amhr2-Cre* recombinase in OSE cells, as demonstrated by LacZ staining of OSE cells in the *Rosa26;Amhr2-Cre* but not in the *Rosa26;Cyp19-Cre* reporter mouse strains (Fig. 4D). These results extend those of Connolly *et al* who generated OSE tumors using the *Amhr2* promoter to direct expression of the SV40 TAg (33) and Szotek *et al* who reported that cell lines derived from mouse ovarian epithelial cell tumors express AMHR2 (34). Furthermore, when the double mutations were made in the *Pgr-Cre* mouse strain, ovaries of the *Pten$^{fl/fl}$;Kras^{G12D};Pgr-Cre* females showed relatively normal histology and were devoid of both the follicle-derived ovarian lesions and OSE cell tumors (Fig. 4D: *Pten$^{fl/fl}$;Kras^{G12D};Pgr-Cre*; right panel) despite the fact that they develop massive uterine tumors and die within 6 weeks after birth (Wang J and DeMayo F, unpublished data).

Due to the expansive growth of OSE cell tumors in the *Pten$^{fl/fl}$;Kras^{G12D};Amhr2-Cre* females, they die between 3-6 months of age (Fig. 5A). In contrast, mice with single mutations of *Pten* (*Pten$^{fl/fl}$;Amhr2-Cre*) were viable (18) and rarely developed GCTs (19) whereas the *Kras^{G12D};Amhr2-Cre* and *Pten$^{fl/fl}$;Kras^{G12D};Cyp19-Cre* females were viable without signs of ovarian tumor development (Fig. 5A), although some OSE cell hyperplasia was observed in the *Kras^{G12D};Amhr2-Cre* females at 10 months or older (data not shown). The abnormal follicle-derived ovarian lesions induced by *Kras^{G12D}* were observed in the *Pten/Kras* double mutant ovaries but the number decreased dramatically (Fig. 5B), suggesting PTEN activity is required to maintain these structures.

PI3K pathway is hyper-activated in *Pten/Kras^{G12D}*-induced OSE tumors

PTEN was expressed in the stromal cells and remnants of abnormal follicle-like structures present in the *Pten$^{fl/fl}$;Kras^{G12D};Cyp19-Cre* ovaries (Fig. 5B). By contrast, the OSE tumor cells present in the *Pten$^{fl/fl}$;Kras^{G12D};Amhr2-Cre* ovaries were completely devoid of PTEN (Fig. 5B) compared to the control OSE cells (Suppl. Fig. 4) and showed elevated activity of the PI3K pathway components compared with wild type controls (Fig. 5C) presumably due to the increased AKT activity (Fig. 5D). Immunofluorescent staining of ovaries from 1 month old *Pten$^{fl/fl}$;Kras^{G12D};Amhr2-Cre* mice showed that AKT and its target kinases mTOR and GSK3β are highly phosphorylated in the invading OSE tumor cells, but not in the non-mitotic granulosa cells (Fig. 5D). The OSE tumor cells showed active proliferation, based on the incorporation of BrdU (Fig. 5D) and immuno-staining of proliferating cell nuclear antigen (PCNA) (Fig. 5D). Strikingly, while expression of KRAS was silenced in granulosa cells of the *Kras^{G12D}*-induced abnormal follicles (Fig 2A), KRAS was high in OSE tumor cells and were associated with high levels of phospho-ERK1/2 and phospho-AKT but not phospho-p38MAPK (Fig. 6A-C; Suppl. Fig. 3D). The inability of OSE cells to inactivate RAS-related signaling events under oncogenic insults may explain why these cells but not granulosa cells are susceptible to tumorigenic stimuli.

Discussion

We document for the first time that the effects of expressing KRAS^{G12D} in ovarian granulosa cells *in vivo* are dependent on the stage of granulosa cell differentiation. In proliferating granulosa cells present in small growing follicles of the *Kras^{G12D};Amhr2-Cre* mice and *Kras^{G12D};Cyp19-Cre* mice, the expression of KRAS^{G12D} led to cell cycle arrest and the absence of genes associated with a differentiated granulosa cell phenotype. As a consequence these mice become infertile. However, in the *Kras^{G12D};Pgr-Cre* mice where the expression of KRAS^{G12D} only occurs in differentiating granulosa of preovulatory follicles after the LH/hCG surge, we observed no overt effects on ovarian function and the mice remained fertile. The consequences of depleting *Pten* in cells expressing KRAS^{G12D} were also cell type specific. Whereas *Kras/Pten* mutant granulosa cells did not exhibit signs of increased proliferation or transformation, OSE cells challenged with the same genetic alterations exhibited serous papillary cystadenocarcinomas within one month of age. These results document unequivocally that the impact of mutant KRAS^{G12D} and *Pten* depletion are cell type specific within the ovary and that granulosa cells, like some other mitotic cells (31,35), possess mechanisms to combat oncogenesis by entering cell cycle arrest (Fig. 6D).

Specifically, our results document in six mutant mouse *in vivo* models that KRAS^{G12D} potently impacts the proliferative capacity of granulosa cells. If recombination occurs in granulosa cells of small growing follicles, these cells become non-mitotic [10] and express extremely low levels of genes and proteins related to cell cycle progression, especially factors regulating entry into S-phase of the cell cycle (27,36) including cyclin A, cyclin D2, and E2F1. Whereas cyclin A and cyclin D2 regulate specific cell cycle dependent kinases, E2F1 regulates the transcription of cyclin A and other transcriptional co-regulators that mediate entry into the S phase (27). Because activin can impact granulosa cell proliferation (37,38) and has been linked to ovarian stromal tumors (39), it may be important that levels of *Inhba* and *Inhbb* are negligible in the *Kras^{G12D}* mutant cells. That the small abnormal follicle-like structures persist and accumulate in the ovaries of the mutant mice is associated with, and may be dependent on the lack of apoptosis as well as cell cycle arrest (10).

The mechanisms regulating cell cycle arrest in the granulosa cells may to be mediated, in part, by the silencing of the *Kras* gene, thus impairing the phosphorylation of ERK1/2 and AKT. Although *Kras^{G12D}* is expressed at the time of recombination (10), the complete absence of KRAS protein in the mutant granulosa cells suggests that *Kras^{G12D}* silences the *Kras* gene. To

our knowledge, the silencing of the *Kras* gene by oncogenic $KRAS^{G12D}$ has not been reported previously, may represent an E2F1 mediated regulatory mechanism (35) and appears to be independent of factors implicated in regulating cell cycle arrest and senescence in other cell types (31,40,41) (Suppl. Fig. 1 and 2). That silencing the *Kras* gene is critical for preventing granulosa cell tumor formation is supported by the observations that the *Pten*$^{fl/fl}$*;Ctnnb1*$^{exon3fl/-}$*; Amhr2-Cre* mice expressing constitutively active beta-catenin (CTNNB1) in the *Pten* null background develop metastatic GCTs with 100% penetrance at an early age (19). Thus, the response of granulosa cells to oncogenic factors depends on which combinations of oncogenic stimuli are expressed in these cells.

Additional mechanisms controlling cell cycle arrest in the *Kras* mutant granulosa cells appear to be associated with and perhaps mediated by elevated levels of the tumor suppressor PTEN, phospho-p38MAPK mediated events and/or the repressor of transcription *Zbtb16* (*Plzf*), all of which are increased in the *Kras*^{G12D} mutant cells. Because PLZF is a potent regulator of germ cell stem cell survival (28,42) and has been linked to cell senescence (43), it may participate in the extended life span and lack of differentiation of these somatic granulosa cells. That phospho-p38MAPK is elevated markedly in the non-mitotic granulosa cells may be important, because phospho-p38MAPK is associated with senescent fibroblasts and epithelial cells (29, 30). Of note, the highly proliferative OSE tumor cells did not exhibit detectable levels of phospho-38MAPK. The mutant granulosa cells within the abnormal follicle-like structures also express markedly elevated levels of PTEN and negligible levels of phospho-AKT indicating that the PI3K pathway is severely inhibited. Although we hypothesized that disrupting *Pten* in the *Kras*^{G12D} mutant strains might prevent cell cycle arrest and lead to granulosa cell tumor formation, the loss of *Pten* in the *Kras*^{G12D} expressing cells did not prevent the formation of the abnormal follicle-like structures. However, the number of abnormal follicle-like structures was reduced and in some ovaries of the double *Kras/Pten* mutant mice regions of the ovary contained luteinized-like structures. Because this ovarian phenotype is more reminiscent of that observed in the *Pten* conditional knockout ovaries [18], it is possible that if the disruption of *Pten* occurs simultaneously with the onset of *Kras*^{G12D} expression, the granulosa cells may luteinize. This is likely because *Kras*^{G12D} expression did not impact ovarian cell function if expressed in luteinizing granulosa cells of the *Pten* $^{fl/fl}$*;Kras*^{G12D}*;PGR-Cre* mice. This agrees with previous reports indicating that *Kras*^{G12D} can cause cell cycle arrest and senescence only in mitotic cells (44) and that the RAS-ERK1/2 pathway is critical for cell cycle arrest during luteinization (45). Remarkably, no GCTs formed in ovaries of the *Pten*$^{fl/fl}$*,Kras*^{G12D}*;Cyp19-Cre* mice even at 10 months of age (data not shown) and were rare in the *Pten* $^{fl/fl}$*;Amhr2-Cre* mice (19).

In marked contrast to the granulosa cells, the OSE cells present in the *Pten*$^{fl/fl}$*;Kras*^{G12D}*;Amhr2-Cre* mouse ovaries transformed rapidly and developed into serous papillary cystadenocarcinomas between 1-3 months of age. That the tumors are derived from OSE cells was confirmed several criteria: LacZ staining of OSE cells only in *Amhr2-Cre* mice and the presence of the epithelial marker cytokeratin 8, an OSE cell marker that has been detected recently in the apical cell membrane of mouse serous adenocarcinoma of the ovary and human ovarian carcinoma, MUC16 (46,47) and a specific marker of human ovarian serous papillary adenocarcinomas, WT-1. The *Kras/Pten* mutant OSE cells expressed elevated levels PI3K pathway components and phospho-ERK1/2, all of which were localized selectively to the invading OSE cells and were not present in the abnormal follicle-like structures. Conversely, the abnormal follicle-like structures contained high levels of phospho-p38MAPKwhereas the mutant OSE did not. Thus, these results combined with those of Connolly *et al* (33) and Szotek *et al* (48) clearly document that the *Amhr2* gene is expressed in normal OSE cells. Although its role in normal, non-transformed cells remains to be clarified, AMH has been shown to block the proliferation of mouse and human ovarian cancer cell lines (48). Thus, the spontaneous and cell specific development of OSE cell tumors in the ovaries

of the *Pten*$^{fl/fl}$,*Kras*^{G12D};*Amhr2Cre* mice described herein provides the first demonstration that *Amhr2-Cre* is expressed in murine OSE cells and can drive oncogenic transformation by generating cell specific mutants. Our results confirm and extend the studies of others who have generated OSE cell tumors by injecting adenoviral-Cre vectors into the ovarian bursa of *Pten*$^{fl/fl}$;*Kras*^{G12D} mice (49) and *Apc*$^{fl/fl}$;*Pten*$^{fl/fl}$ mice (50). Furthermore, our results document that the OSE cells in the *Pten*$^{fl/fl}$,*Kras*^{G12D};*Amhr2-Cre* mice develop tumors whereas the granulosa cells do not.

In summary, these studies document that expression of *Kras*^{G12D} and disruption of the *Pten* gene in granulosa cells results in a phenotype distinct from that occurring in OSE cells present in the same ovary (Fig. 6D). Whereas expression of KRAS^{G12D} leads to cell cycle arrest in granulosa cells where the *Kras* gene is silenced, it promotes proliferation in OSE cells where KRAS remains elevated. When *Pten* is disrupted in the *Kras*^{G12D} strain, it enhances OSE cell proliferation and tumor formation, at least in part, by the sustained phosphorylation and activation of ERK1/2 and PI3K pathway components and perhaps suppression of p38MAPK activation. Conversely, disrupting *Pten* in the *Kras* mutant granulosa cells does not reverse cell cycle arrest or induce tumorigenesis of these cells (Fig. 6D). By understanding how KRAS^{G12D} can exert completely opposite effects in these two cell populations within the same tissue may provide a key for controlling cancer development and progression. One key may be the total silencing of the *Kras* gene itself.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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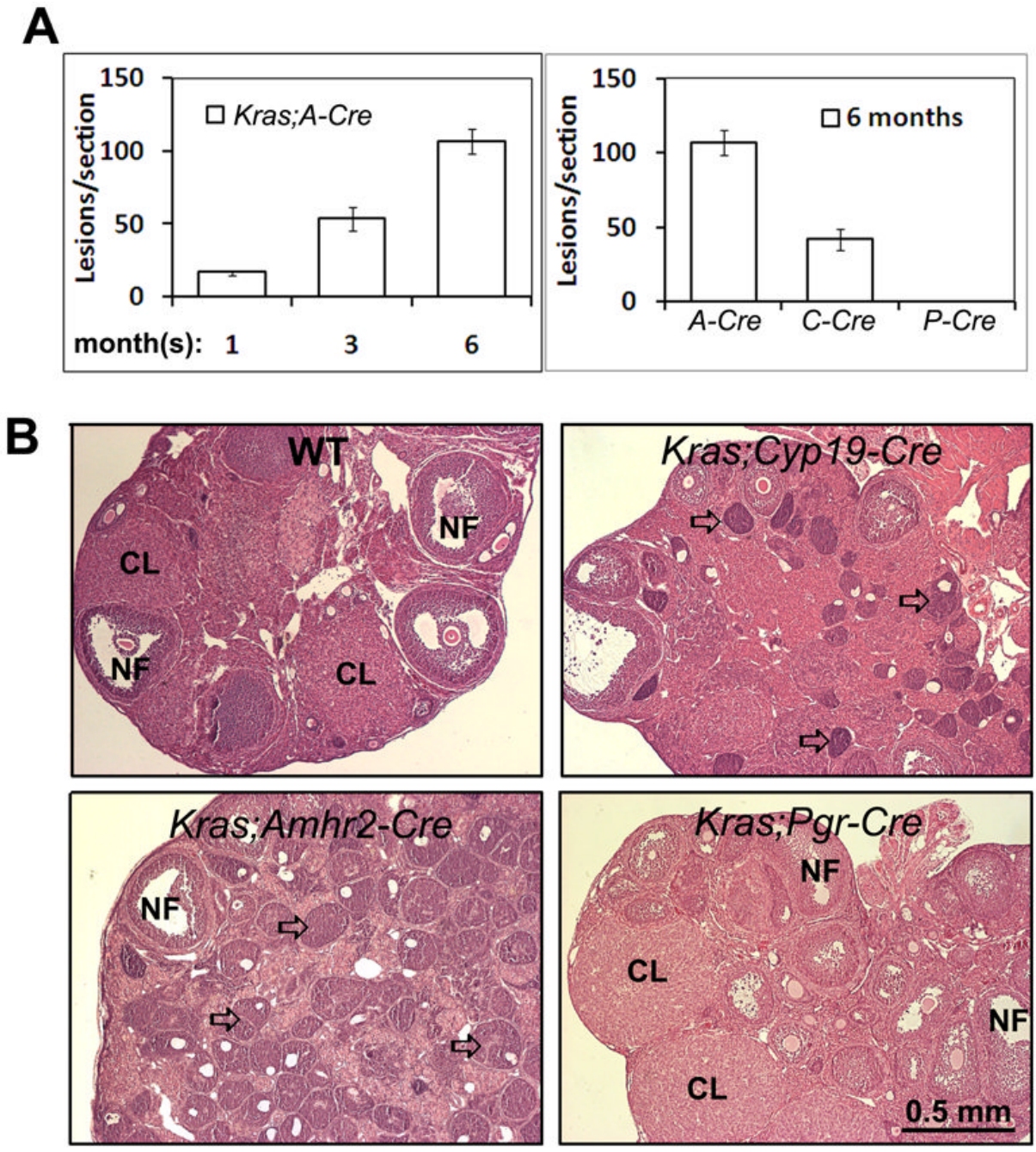

Figure 1. *Kras*^{G12D} selectively impacts granulosa cell function in mice expressing Cre driven by the *Amhr2* (A-Cre) and *Cyp19* (C-Cre) but not *Pgr* (P-Cre) promoters

Ovaries of *Kras*^{G12D}*;A-Cre* mice accumulate more small abnormal follicle-like lesions (arrows) from 1-6 months of age than did the *Kras*^{G12D}*;C-Cre* mice (A &B). No abnormal follicles were observed in ovaries of the *Kras*^{G12D}*;Pgr-Cre* mice. NF: Normal follicles; CL: corpora luteum.

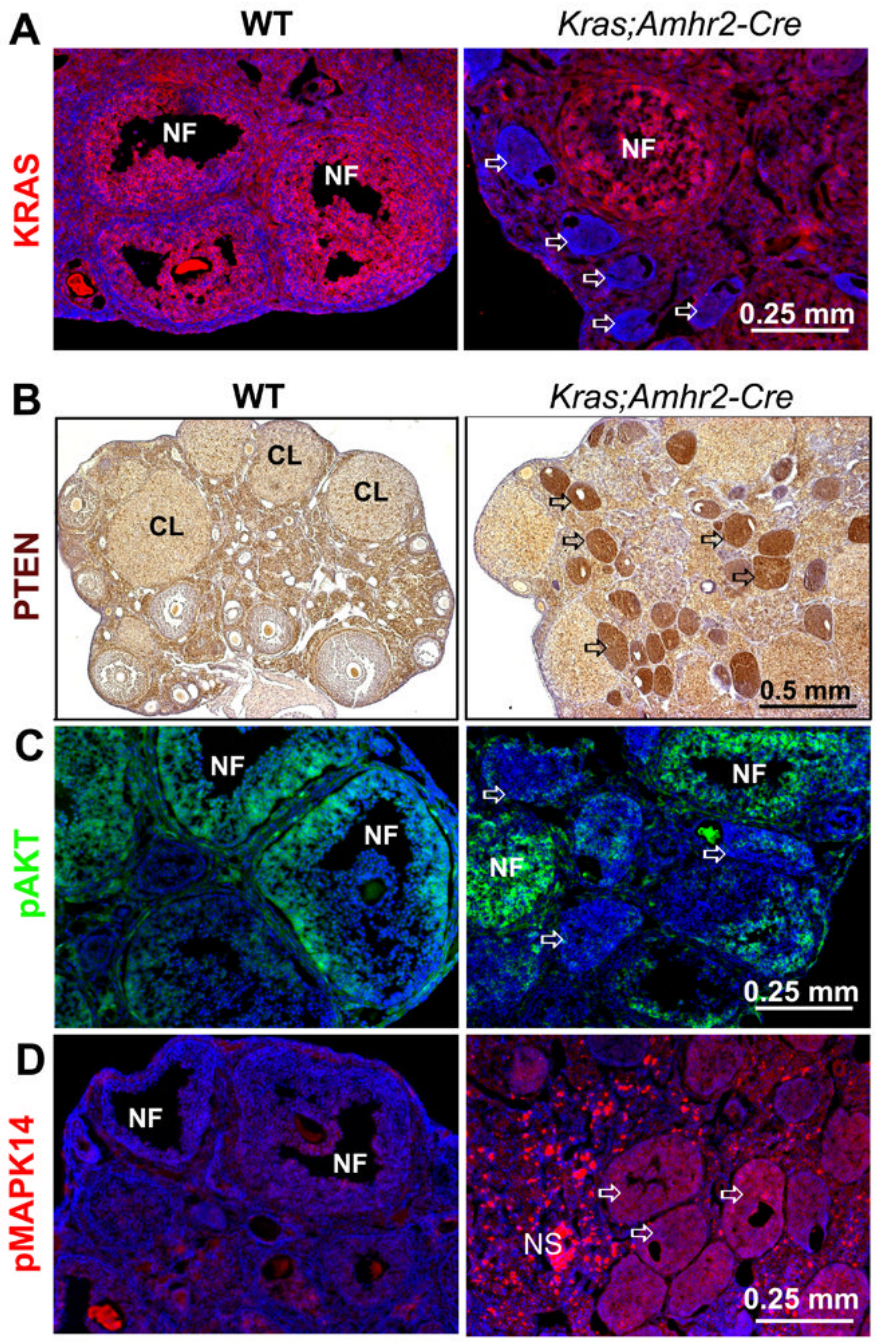

Figure 2. ***Kras*^{G12D} down-regulates KRAS and up-regulates PTEN in the follicle-like lesions**

Granulosa cells of normal growing follicles express KRAS whereas KRAS was negligible in cells within the abnormal follicles (A, arrows). NF: normal follicles. PTEN levels were low in granulosa cells of normal follicles but were elevated in the mutant granulosa cells (B, arrows) and this was associated with reduced levels of phospho-AKT (wild type versus *Kras*^{G12D}*;A-Cre*: (C) but high levels of pMAPK (phospho-p38MAPK) (D).

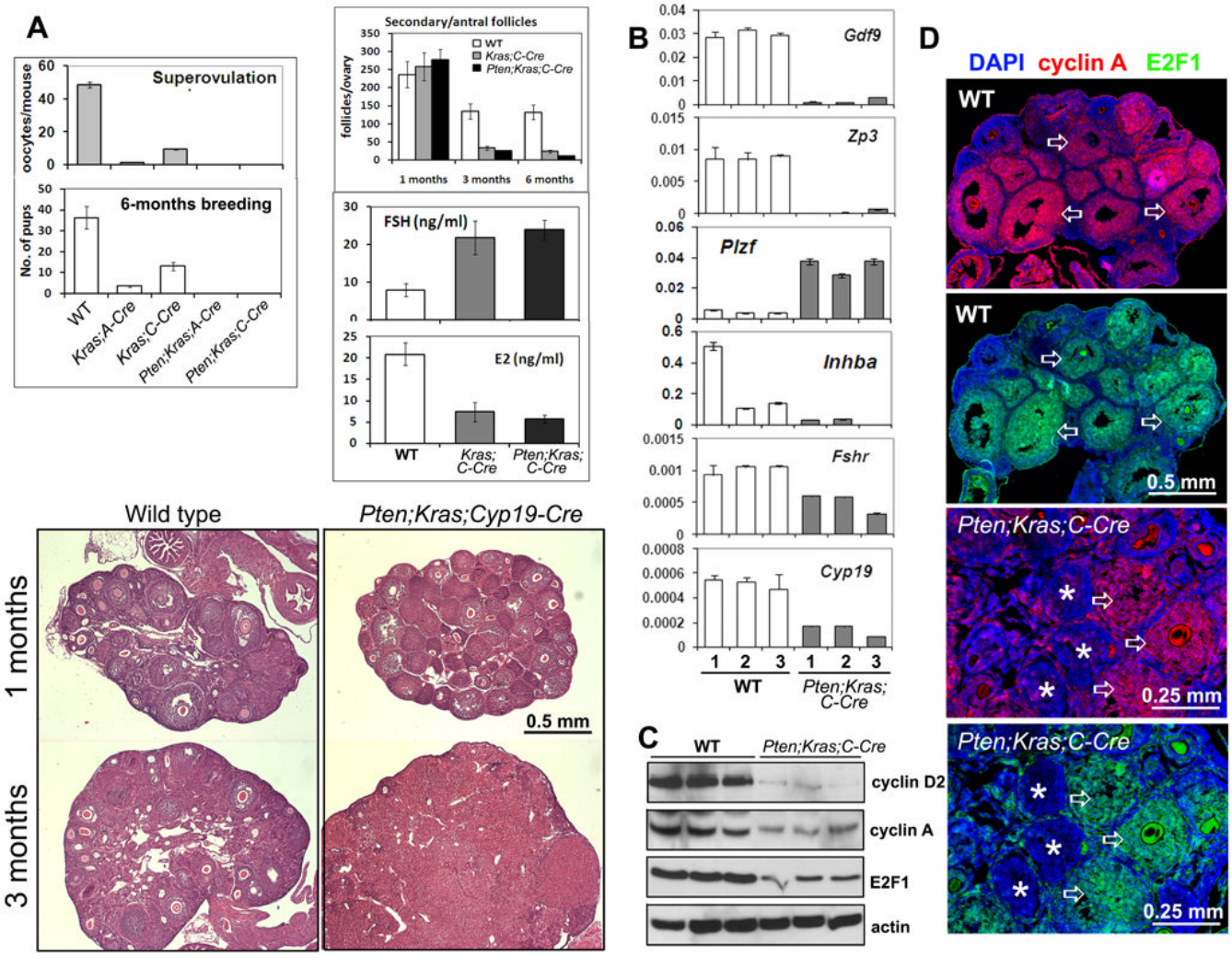

Figure 3. *Pten/Kras* double mutant mice exhibit signs of premature ovarian failure (POF)

Fertility of females in the various mouse strains was evaluated by super-ovulation and breeding assays (A). *Kras* and *Pten/Kras* double mutant mice have reduced numbers of ovarian follicles, high serum FSH and low serum estradiol, symptoms of POF (A). H&E staining of ovarian sections confirmed the altered ovarian phenotype of *Pten/Kras* mutant mice at 1 and 3 months of age (A). Genes crucial for oocyte function and follicle development were down-regulated in *Pten/Kras* double mutant ovaries compared to wild-type at 3 months of age (B). Cell cycle regulators were decreased in *Pten/Kras* double mutant ovaries (C). Cyclin A (red) and E2F1 (green) are highly expressed in granulosa cells present in growing follicles (arrows) in wildtype (WT) and $Pten^{fl/fl}$;$Kras^{G12D}$;*Cyp19-Cre* mice, but not in cells present in abnormal follicles (asterisks) of 1 month old $Pten^{fl/fl}$;$Kras^{G12D}$;*Cyp19-Cre* mice (D).

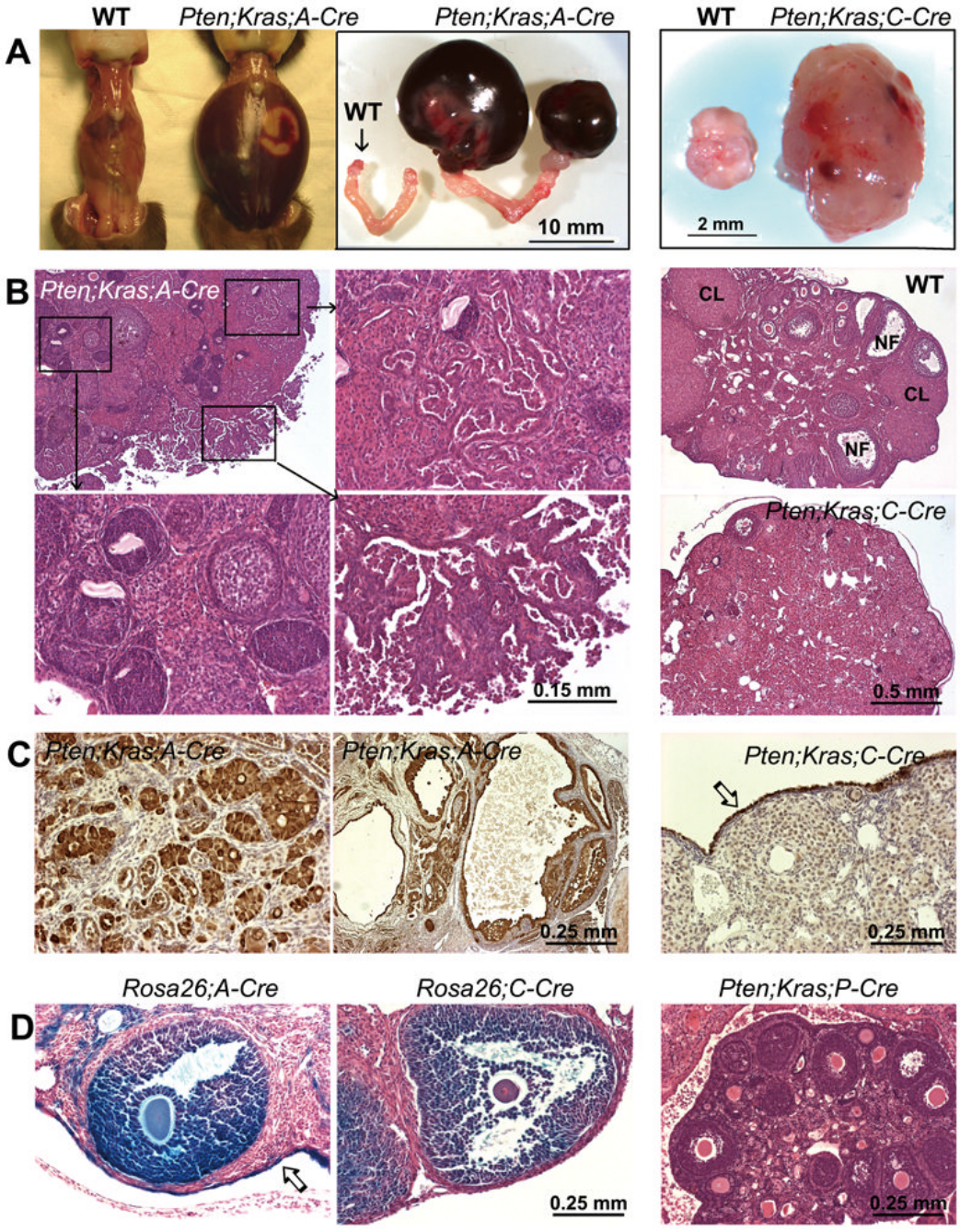

Figure 4. OSE (OSE) cell cancers develop in the *Pten$^{fl/fl}$;Kras^{G12D};Amhr2-Cre* mice but NOT in the *Pten$^{fl/fl}$;Kras^{G12D};Cyp19-Cre* mice or *Pten$^{fl/fl}$;Kras^{G12D};Pgr-Cre* mice

Pten$^{fl/fl}$;Kras^{G12D};Amhr2-Cre female mice develop massive low grade serous, papillary cystadenocarcinomas by 3 months of age (A - C). Multifocally the cystadenocarcinoma invades the ovarian parenchyma (Panel B, upper right) and surround the abnormal follicle-like structures (Panel B; lower left). Ovaries of the *Pten$^{fl/fl}$;Kras^{G12D};Cyp19-Cre* mice are larger ovaries than wild type mice due to the hyperplasia of interstitial tissue, but do not develop ovarian tumors (Panels A-C; right). The epithelial cell origin of tumors was confirmed by immuno-staining of cytokeratin 8 (CK8) in wild-type and *Pten$^{fl/fl}$;Kras^{G12D};Cyp19-Cre* mice (Panel C; right) and in tumor structures invading the ovary (Panel C; left) as well as in tumor-

derived cysts (Panel C; middle). LacZ- staining in *Rosa26* reporter mouse strains showing localization to granulosa cells in *Cyp19-Cre* mice (D; middle panel) and to OSE cells in *Amhr2-Cre* mice (D; left panel). Ovaries from a 5 week old $Pten^{fl/fl};Kras^{G12D};Pgr$-*Cre* mice have no abnormal follicle-like lesions and no OSE tumors (D; right panel), despite the development of massive uterine tumors (data not shown).

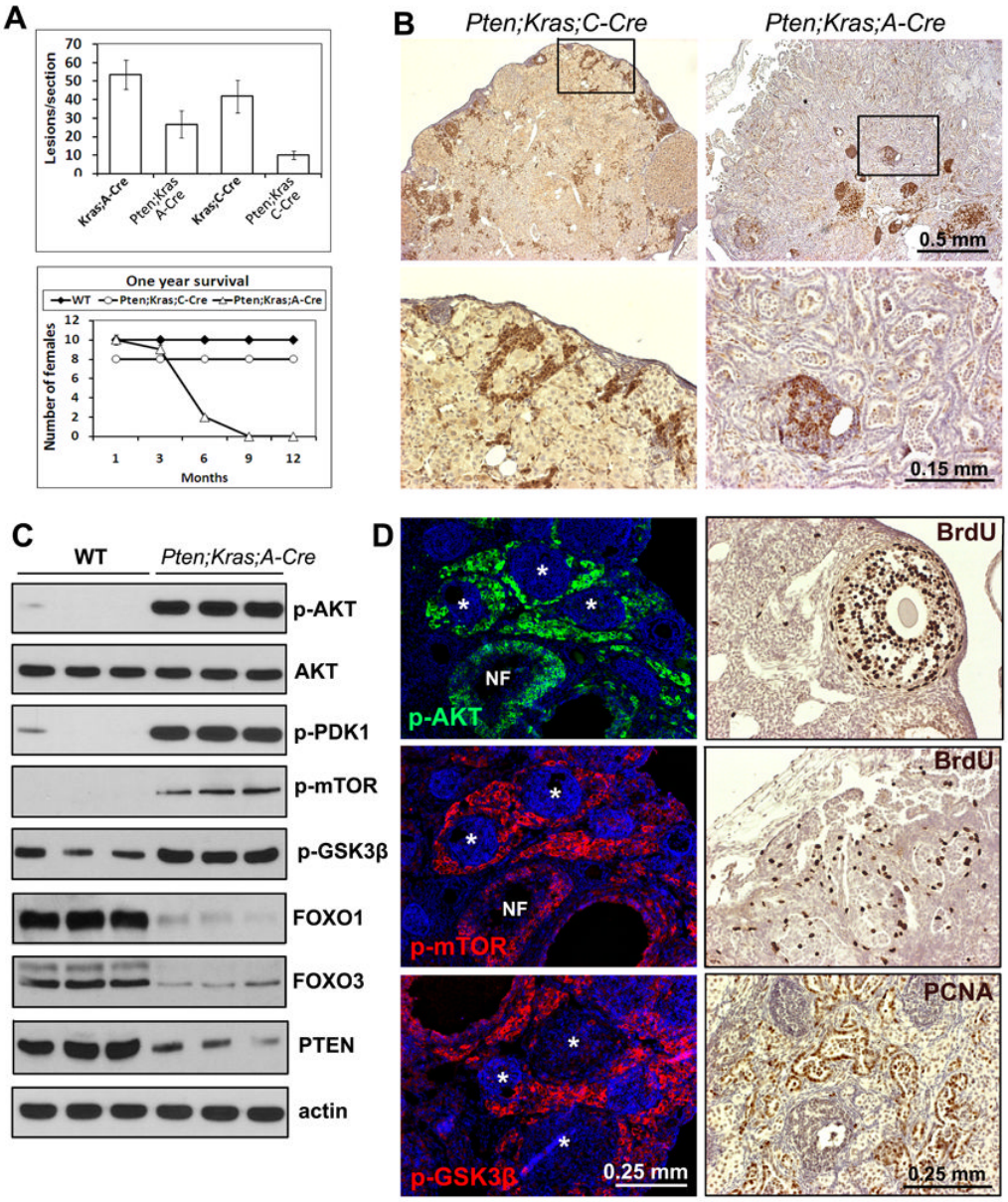

Figure 5. The PI3K pathway is hyper-activated in *Pten/Kras*-induced OSEtumors

Conditional depletion of *Pten* in granulosa cells reduced the numbers of ovarian lesions in ovaries of both *Pten*$^{fl/fl}$;*Kras*^{G12D};*Amhr2-Cre* mice and *Pten*$^{fl/fl}$;*Kras*^{G12D};*Cyp19-Cre* mice (A). Most *Pten*$^{fl/fl}$;*Kras*^{G12D};*Amhr2-Cre* mice died of ovarian cancers between 3-6 months of age (A). PTEN was expressed in the ovarian stroma of *Pten*$^{fl/fl}$;*Kras*^{G12D};*Cyp19-Cre* mice (B) but was absent in OSE tumor cells of *Pten*$^{fl/fl}$;*Kras*^{G12D};*Amhr2-Cre* mice (B). Western blot analyses (C) and immunofluorescent staining (D; left panels) of PI3K pathway components in OSE cells. BrdU is incorporated into proliferating granulosa cells in normal (WT) ovaries (D; upper right panel) and the OSE tumor cells in *Pten*$^{fl/fl}$;*Kras*^{G12D};*Amhr2-Cre* mice (D; middle right panel). The OSE tumor cells also stained for PCNA (D; lower right panel).

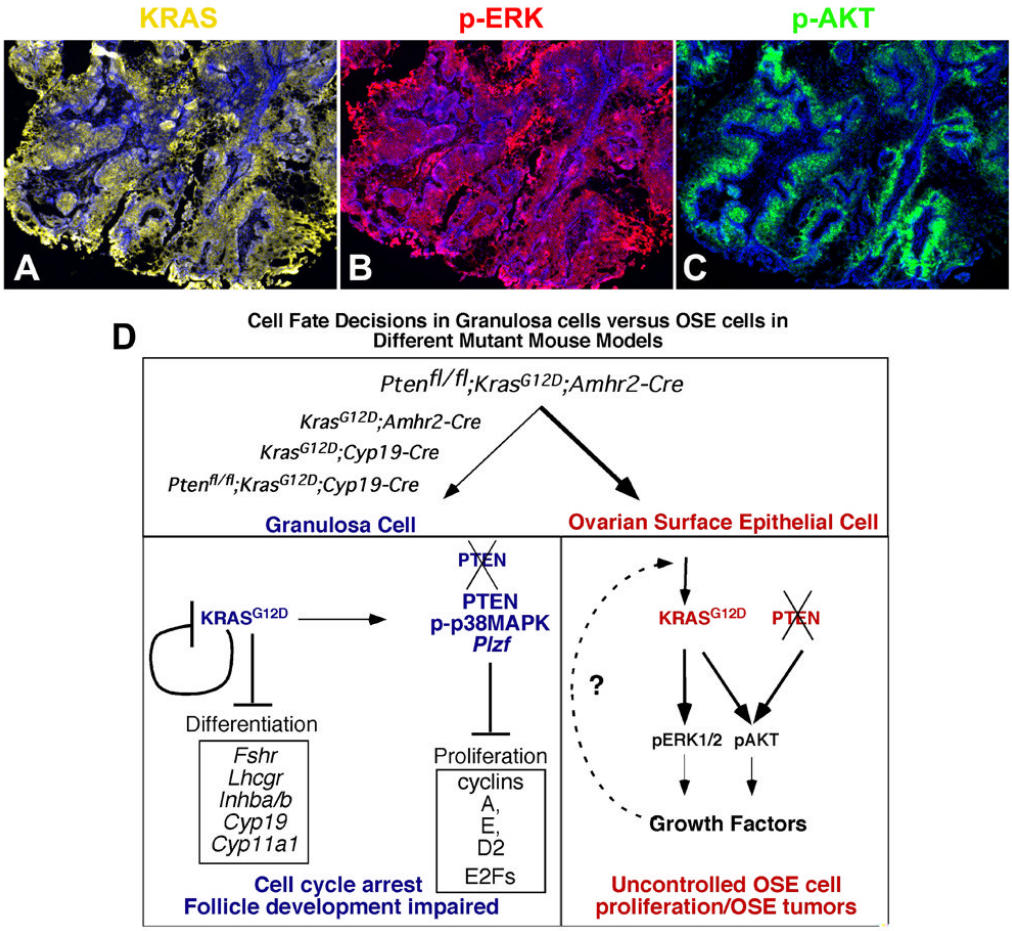

Figure 6. KRAS-related signaling pathways are actively involved in the development of OSEcancers

KRAS is highly expressed by OSE tumor cells of *Pten*$^{fl/fl}$;*Kras*^{G12D};*Amhr2-Cre* mice (A) and this is associated with elevated levels of phospho- ERK1/2 (B) and phospho-AKT (C). The cell and stage specific effects of *Kras/Pten* mutations on OSE cells and granulosa cells were summarized schematically (D).