Synergistic effects of *Pten* loss and WNT/CTNNB1 signaling pathway activation in ovarian granulosa cell tumor development and progression

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The mechanisms of granulosa cell tumor (GCT) development may involve the dysregulation of signaling pathways downstream of follicle-stimulating hormone, including the phosphoinosite-3 kinase (PI3K)/AKT pathway. To test this hypothesis, a genetically engineered mouse model was created to derepress the PI3K/AKT pathway in granulosa cells by conditional targeting of the PI3K antagonist gene *Pten* (*Pten*flox/flox;*Amhr2*cre/+). The majority of *Pten*flox/flox;*Amhr2*cre/+ mice featured no ovarian anomalies, but occasionally (~7%) developed aggressive, anaplastic GCT with pulmonary metastases. The expression of the PI3K/AKT downstream effector FOXO1 was abrogated in *Pten*flox/flox;*Amhr2*cre/+ GCT, indicating a mechanism by which GCT cells may increase proliferation and evade apoptosis. To relate these findings to spontaneously occurring GCT, analyses of PTEN and phospho-AKT expression were performed on human and equine tumors. Although PTEN loss was not detected, many GCT (2/5 human, 7/17 equine) featured abnormal nuclear or perinuclear localization of phospho-AKT, suggestive of altered PI3K/AKT activity. As inappropriate activation of WNT/CTNNB1 signaling causes late-onset GCT development and cross talk between the PI3K/AKT and WNT/CTNNB1 pathways has been reported, we tested whether these pathways could synergize in GCT. Activation of both the PI3K/AKT and WNT/CTNNB1 pathways in the granulosa cells of a mouse model (*Pten*flox/flox;*Ctnnb1*flox(ex3)/+;*Amhr2*cre/+) resulted in the development of GCT similar to those observed in *Pten*flox/flox;*Amhr2*cre/+ mice, but with 100% penetrance, perinatal onset, extremely rapid growth and the ability to spread by seeding into the abdominal cavity. These data indicate a synergistic effect of dysregulated PI3K/AKT and WNT/CTNNB1 signaling in the development and progression of GCT and provide the first animal models for metastatic GCT.

Introduction

The granulosa cell tumor (GCT) is the most common neoplastic disease of the ovary in most domestic species (1). In women, GCT is the
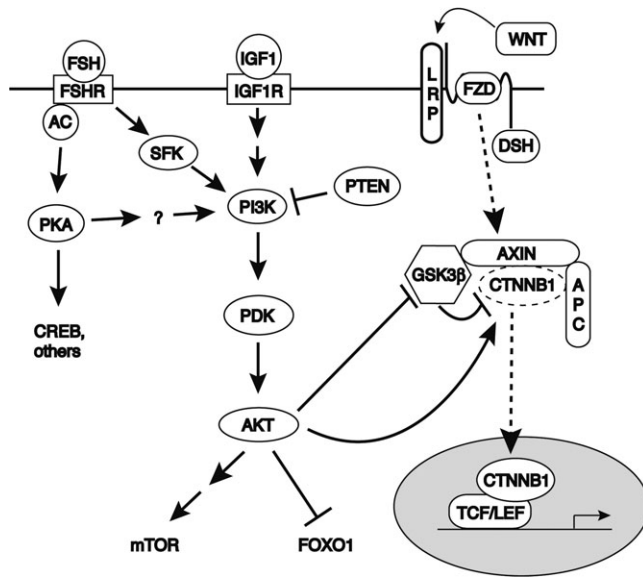
most prevalent of the sex cord/stromal tumors and is thought to represent up to 5% of all ovarian cancers (2). Although GCT is often characterized as a low-grade malignancy, a large proportion of patients develop recurrences as late as 40 years after the initial diagnosis and treatment, and therefore fastidious long-term follow-up is required (3,4).

Despite the importance and insidiousness of GCT, very little is known of its molecular etiology. Only a handful of reports have identified genetic or molecular lesions in GCT and have generally provided little insight into the mechanisms of GCT development (5–8). Transgenic mouse models that overexpress luteinizing hormone (9) and that express SV40 T-antigen in their granulosa cells (10,11) and *Inha* knockout mice (12) all develop GCT; however, it remains unclear if the molecular mechanisms of tumorigenesis in these animal models are related to those involved in GCT development in women and other species. Recently, we have reported evidence that the WNT/CTNNB1 signaling pathway is dysregulated in many GCT and that transgenic mice featuring constitutive activation of the WNT/CTNNB1 pathway in their granulosa cells (*Ctnnb1*flox(ex3)/+;*Amhr2*cre/+) developed GCT with many histological similarities to the human disease (5). Interestingly, peripubertal *Ctnnb1*flox(ex3)/+;*Amhr2*cre/+ mice developed multiple pretumoral ovarian lesions consisting of follicle-like nests of granulosa cells whose growth was self-limiting (5,13). Progression of these pretumoral lesions into GCT occurred in a stochastic manner after the age of 5 months, suggesting that while the activation of the WNT/CTNNB1 pathway in granulosa cells induces a premalignant state, it is rarely (if ever) sufficient by itself to cause GCT. Therefore, while these data represented a significant advance in our understanding of the molecular etiology of GCT, it is clear that additional factors and pathways must be involved.

A logical and frequently taken approach to identify genes involved in the etiology of GCT has been to search for mutations in genes normally involved in granulosa cell proliferation (6). As follicle-stimulating hormone (FSH) is a major growth factor of granulosa cells, several groups have sought to identify activating mutations in *Fshr* (14–16,17) or in G-protein subunits involved in transducing the FSH signal (18–20), but to little avail. FSH signal transduction is thought to be mediated mainly via the protein kinase A pathway (21), but a recent series of reports have revealed that FSH signaling is much more complex than previously suspected and involves the activation of several signaling pathways (22). Notably, FSH can activate the phosphoinosite-3 kinase (PI3K)/AKT pathway through a mechanism that involves SRC family protein kinases and may or may not involve protein kinase A (Figure 1) (22–25). Some of the molecular events downstream of PI3K/AKT activation by FSH in granulosa cells have been elucidated and include the phosphorylation and nuclear export of the Forkhead family transcription factor FOXO1 and the activation of the protein kinase mammalian target of rapamycin (mTOR) (Figure 1) (22,24). These events lead in turn to the modulation of the expression of FSH target genes, including *Ccnd2* and *Hif1a* (22). In addition to transducing a variety of physiological signals, the PI3K/AKT pathway is also known to contribute to the development of a large number of cancers when dysregulated, and many components of this pathway function either as tumor suppressor genes or proto-oncogenes (6,26–28). Perhaps most notably, the tumor suppressor gene *PTEN* attenuates PI3K/AKT pathway activity by acting as a lipid phosphatase on phosphatidylinositol (3,4,5)-trisphosphate and is frequently inactivated in several forms of cancer (28). Although the aforementioned data clearly indicate the potential involvement of the PI3K/AKT pathway in GCT development, no study of PI3K/AKT signaling in GCT has been reported thus far.

Another well-known downstream effector of the PI3K/AKT pathway is the protein kinase glycogen synthase kinase-3β (GSK3β) (29,30). Activated AKT inhibits GSK3β by phosphorylation of an

Fig. 1. Schematic representation of cross talk between PKA, PI3K/AKT and WNT/CTNNB1 intracellular signaling pathways. AC = adenylyl cyclase, APC = adenomatosis polyposis coli, CREB = cAMP response element binding, DSH = disheveled, FOXO1 = forkhead box O1, FSH(R) = follicle stimulating hormone (receptor), FZD = frizzled, IGF1(R) = insulin-like growth factor-1 (receptor), LRP = low-density lipoprotein receptor-related protein, PDK = 3-phosphoinositide-dependent protein kinase, PI3K = phosphoinositide 3-kinase, PKA = protein kinase A, PTEN = phosphatase and tensin homolog, SFK = SRC family protein kinases, TCF/LEF = transcription factor, T-cell specific/lymphoid enhancer factor 1 and CTNNB1 = β-catenin.

N-terminal serine residue, thereby modulating various cellular processes including glycogen metabolism (31). One substrate of GSK3β is CTNNB1, a multifunctional protein that is both a structural component of cell–cell adhesion structures and an important signal transduction effector that is activated by the WNT family of signaling molecules (32). GSK3β is a negative regulator of CTNNB1 and acts by phosphorylating a series of N-terminal serine and threonine residues, resulting in the later ubiquitination and degradation of CTNNB1 by the cellular proteosomal machinery (32). Phosphorylation of GSK3β by AKT therefore results in the hypophosphorylation, stabilization and accumulation of CTNNB1, which subsequently translocates to the cell nucleus and associates with various transcription factors to modulate the transcriptional activity of specific target genes (Figure 1). In addition to this indirect activation mechanism, AKT has recently been shown to directly phosphorylate CTNNB1 at a distinct site, resulting in an increase in its transcriptional activity (33). Importantly, the transduction of WNT signaling also involves hypophosphorylation and stabilization of CTNNB1 (32). GSK3β/CTNNB1 therefore represents an important point of convergence and cross talk between the PI3K/AKT and WNT/CTNNB1 pathways (Figure 1). Indeed, PI3K/AKT signaling is believed to employ the WNT/CTNNB1 pathway in this manner in several physiological and developmental contexts (34–38), as well as in the development and progression of several forms of cancer, including those of the mammary gland, prostate, liver and skin (30,39–44). Although dysregulated WNT/CTNNB1 signaling is clearly involved in the pathogenesis of GCT (5,13), whether the PI3K/AKT and WNT/CTNNB1 pathways can interact or synergize in GCT development or in normal physiological contexts in ovarian granulosa cells remains unknown.

The objective of this study was to investigate whether dysregulated PI3K/AKT signaling could be involved in GCT development. Transgenic *Pten*^flox/flox;*Amhr2*^cre/+ mice were created to constitutively derepress PI3K/AKT signaling in granulosa cells *in vivo*. *Pten*^flox/flox;*Amhr2*^cre/+ mice occasionally developed aggressive and metastatic GCT, demonstrating the importance of *Pten* loss in GCT

development. Furthermore, concurrent *Pten* loss and activation of the WNT/CTNNB1 pathway in the granulosa cells of a second mouse model, *Pten*^flox/flox;*Ctnnb1*^flox(ex3)/+;*Amhr2*^cre/+, resulted in the development of an even more aggressive GCT phenotype with 100% penetrance, demonstrating a synergistic effect of the genetic lesions. This study therefore offers important new insights into the etiology of GCT and provides the first model systems for metastatic GCT.

Materials and methods

Animals and genotype analyses

Genetically modified animals were derived by selective breeding of the previously described *Pten*^flox, *Ctnnb1*^flox(ex3) and *Amhr2*^cre parental strains (45–47). Genotyping analyses for the *Amhr2*^cre and *Ctnnb1*^flox(ex3) alleles were performed by polymerase chain reaction (PCR) on DNA obtained from tail biopsies as described previously (47,48). Mice bearing the *Pten*^flox allele were obtained from The Jackson Laboratory (Bar Harbor, ME), and DNA samples from tail biopsies, tumor samples or isolated whole ovaries were analyzed for *Pten* genotype by PCR as directed by the animal provider, except the following oligonucleotide primers were used: 5′-GATACTAGTAAGATAAAAACCAG-TAGT-3′, 5′-GTCACCCAGGCCTCTTGTCAAGT-3′ and 5′-GCTTGA-TATCGAATTCCTGCAGC-3′. These primers produce PCR products of ~400 bp for the wild-type *Pten* allele, 572 bp for the floxed allele and 290 bp for the Cre-recombined allele. All animal procedures were approved by the Institutional Animal Care and Use Committee and were conform to the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded, 7 μm tissue sections using VectaStain Elite avidin–biotin complex methods kits (Vector Labs, Burlingame, CA) as directed by the manufacturer, except incubation with primary antibodies was performed overnight at 4°C. The Vector M.O.M. Basic Kit (Vector Labs, Burlingame, CA) was used whenever a mouse-derived primary antibody was applied to mouse tissues. Sections were probed with primary antibodies against PTEN or phospho(Ser473)-AKT (Cell Signaling Technology, Danvers, MA, catalog numbers 9559 and 4051, respectively) using the manufacturers suggested conditions. Staining was performed using the 3,3′-diaminobenzidine peroxidase substrate kit (Vector Labs) as directed, and slides were lightly counterstained with hematoxylin prior to mounting. Human and equine GCT samples consisted of archived, paraffin-embedded tumor fragments obtained during surgical resections at the University of Texas M.D. Anderson Cancer Center (Houston, TX), Hôpital Maisonneuve-Rosemont (Montréal, Québec, Canada) and the Centre Hospitalier Universitaire Vétérinaire de l′Université de Montréal (St Hyacinthe, Québec, Canada). Normal human ovary samples (n = 4) were obtained from the same establishments as the GCT and were removed from premenopausal women undergoing surgery for non-ovarian gynecologic conditions. Preovulatory equine follicular samples (n = 3) were obtained as described previously (49).

Semiquantitative reverse transcription–PCR

Tissue samples used for reverse transcription–PCR analyses were distinct from those used for immunohistochemistry and were obtained as described previously (50,51). Reverse transcription was performed on 1 μg RNA samples derived from human GCT or normal ovary as described previously (50,51). PCR was then performed on 1% of the resulting complementary DNA samples using the oligonucleotide primers 5′-CATTTGCAGTATAGAGCGTGCAGA-3′ and 5′-TGTATGCTGATCTTCATCAAAAGGT-3′ for *PTEN* and 5′-GGAAGGTGAAGGTCGGGAGTCAA-3′ and 5′-CCAGCCTTCTCCATG-GTGGTGA-3′ for *GAPDH*. Cycling conditions were 94°C for 1 min followed by 25 (*GAPDH*) or 30 cycles (*PTEN*) of 94°C for 30 s, 60°C for 1 min and 72°C for 1 min. Preliminary experiments were performed for *PTEN* and *GAPDH* to ensure that the cycle numbers selected fell within the linear range of PCR amplification (data not shown). A complementary DNA sample produced from luteinizing human granulosa cells isolated from patients undergoing *in vitro* fertilization procedures (a kind gift from Dr Bruce Murphy, Université de Montréal, Québec, Canada) was analyzed as for the GCT and ovarian samples described above. PCR products were separated by electrophoresis on 2% tris-acetate-EDTA-agarose gels containing ethidium bromide and photographed under ultraviolet light.

Immunoblotting

Granulosa cells were obtained for immunoblotting analysis from 20- to 26-day-old *Pten*^flox/flox or *Pten*^flox/flox;*Amhr2*^cre/+ animals that had been given eCG (Folligon, Intervet, Whitby, Canada, 5 IU, intraperitoneally) 48 h prior to

sacrifice, and cells were isolated using the needle puncture method as described previously (52). Granulosa cells from three mice were pooled to create samples of sufficient size for analyses, and protein extracts were obtained using M-PER mammalian protein extraction reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. *Pten*flox/flox;*Amhr2*cre/+ GCT protein samples were prepared as described previously (53), and all protein samples concentrations were quantified using the Bradford method. Samples (50 µg) were resolved on 7.5–15% sodium dodecyl sulfate–polyacryl-amide gels and transferred to Hybond-P PVDF membrane (GE Amersham, Piscataway, NJ). Blots were then probed with antibodies against AKT, FOXO1, phospho(Ser256)-FOXO1, mTOR, phospho(Ser2448)-mTOR, GSK3β and phospho(Ser9)-GSK3β (Cell Signaling catalog numbers 9272, 9462, 9461, 2972, 2971, 9315 and 9323, respectively), β-ACTIN (Santa Cruz Biotechnol-ogy, Santa Cruz, CA, catalog number sc-47778), PTEN or phospho-AKT (described above) as directed by the manufacturer. Following incubation with horseradish peroxidase-conjugated secondary antibody (GE Amersham), the protein bands were visualized by chemiluminescence using the ECL Plus Western Blotting Detection Reagents (GE Amersham) and High-Performance Chemiluminescence film (GE Amersham).

Statistical methods

Effects of genotype on number of concepti were analyzed by unpaired, two-tailed *t*-test. *P*-values <0.05 were considered statistically significant. Analyses were performed using Prism 4.0a software (GraphPad Software, San Diego, CA).

Results

Lack of ovarian anomalies in most Pten*flox/flox*;Amhr2*cre/+* mice

To study the potential role of dysregulated PI3K/AKT signaling in GCT development, a conditional gene targeting strategy was devised to constitutively derepress the PI3K/AKT pathway in the granulosa cells of mice. Mice bearing a floxed *Pten* allele (45) were mated to a strain in which the *Cre* transgene had been knocked in to the *Amhr2* locus (46). The resulting *Pten*flox/flox;*Amhr2*cre/+ mice would therefore be predicted to bear null mutations of *Pten* in their granulosa cells, resulting in derepression of PI3K/AKT pathway activity. Unexpectedly, the vast majority of female *Pten*flox/flox;*Amhr2*cre/+ mice showed no morphological or functional ovarian anomalies. Histopathological examination of ovaries between the time of birth and 1 year of age revealed no differences between *Pten*flox/flox;*Amhr2*cre/+ and *Pten*flox/flox controls (Figure 2A and data not shown). Furthermore, *Pten*flox/flox; *Amhr2*cre/+ females could establish pregnancies, and no significant differences in numbers of concepti were noted by day 9.5 post-coitum relative to controls (7.0 ± 0.58 in *Pten*flox/flox;*Amhr2*cre/+ versus 7.5 ± 0.65 *Pten*flox/flox, *n* = 4, mean ± SEM, *P* > 0.05), suggesting that follicle development and growth, ovulation and formation and func-tion of the corpus luteum were all normal in *Pten*flox/flox;*Amhr2*cre/+ mice. However, many *Pten*flox/flox;*Amhr2*cre/+ mice failed to carry pregnancies to term or had small litters due to fetal death after day 9.5 post-coitum. Serum progesterone measurements throughout preg-nancy failed to show differences between *Pten*flox/flox;*Amhr2*cre/+ mice and controls (M.N.Laguë, J.S.Richards and D.Boerboom, unpub-lished observations), suggesting that fetal loss was most probably due to an extra-ovarian defect.

To explain the lack of an ovarian phenotype in *Pten*flox/flox;*Amhr2*cre/+ mice, the efficiency of the *Cre*-mediated genetic recombination pro-cess was assessed by PCR genotyping analyses performed on DNA isolated from whole ovaries. Results showed the presence of low but detectable levels of the recombined *Pten* allele in ovarian DNA from *Pten*flox/flox;*Amhr2*cre/+ mice, but not from *Pten*flox/flox controls (Figure 2B). This low efficiency of genetic recombination resulted in no appreciable changes in PTEN or phospho-AKT protein levels in *Pten*flox/flox;*Amhr2*cre/+ granulosa cells, as determined by immunoblot-ting (Figure 2C and data not shown). However, immunohistochemical analyses revealed the presence of rare granulosa cells that expressed elevated levels of phospho-AKT, which were not observed in the ova-ries of *Pten*flox/flox controls (Figure 2D). Together, these results indi-cated that recombination of the floxed *Pten* allele was inefficient in *Pten*flox/flox;*Amhr2*cre/+ granulosa cells and was not sufficient to induce ovarian granulosa cell tumorigenesis in most cases.

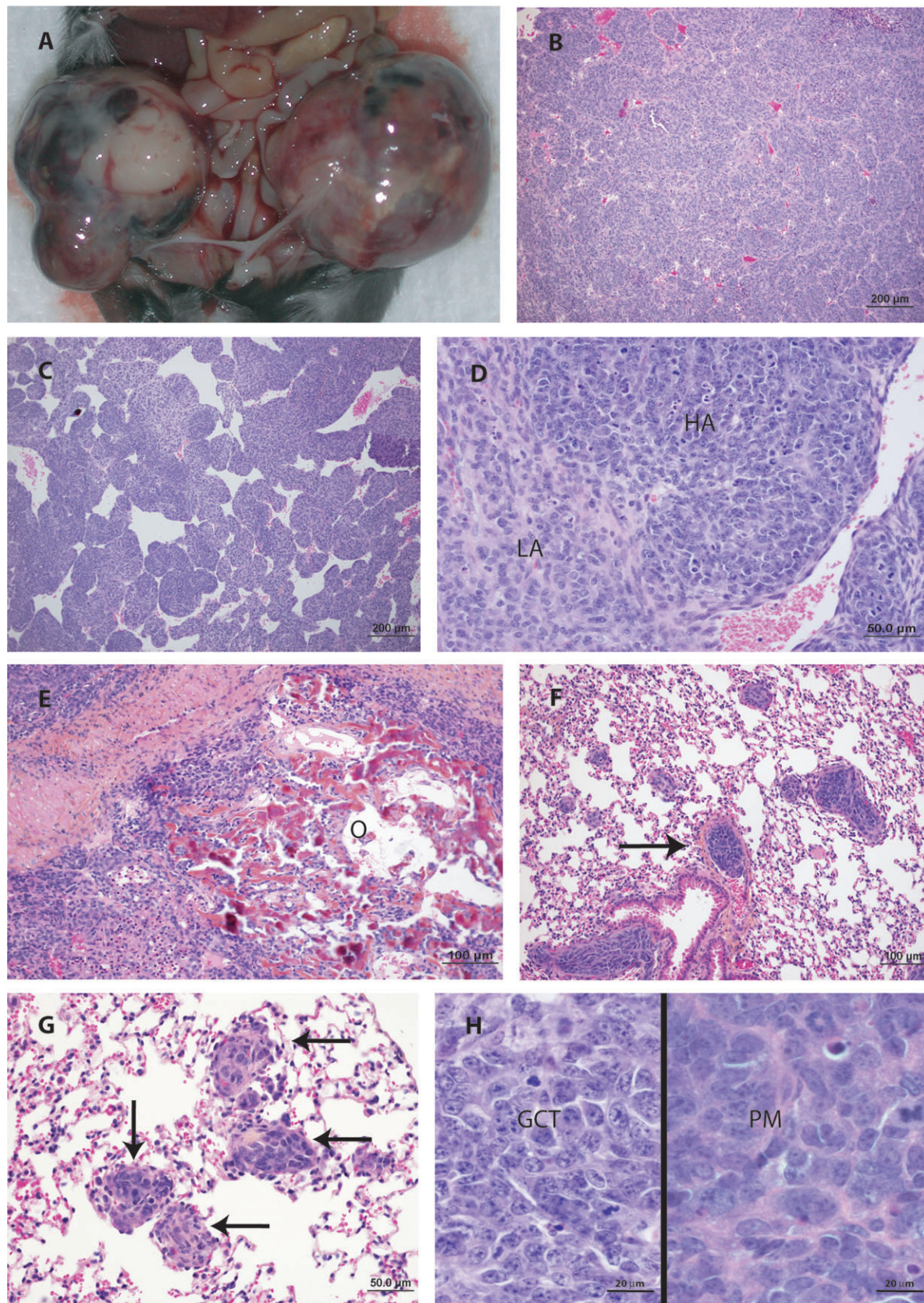### Rare Pten*flox/flox*;Amhr2*cre/+* mice develop aggressive GCT

Despite the apparent lack of consequence of the loss of *Pten* expres-sion in most *Pten*flox/flox;*Amhr2*cre/+ mice, five of the 70 (~7%) female *Pten*flox/flox;*Amhr2*cre/+ mice that we generated in the context of this study developed ovarian tumors (Figure 3A). Of the five animals, two were diagnosed postmortem, having presumably died from the effects of the tumors. Tumors were bilateral in four of the five mice, ages at diagnosis varied from 7 weeks to 7 months and all but one mouse were virginal. Histopathological examination of the tumors revealed them to be GCT. The cells within the GCT were arranged in either a solid or trabecular pattern, and both patterns were found in most tumors (Figure 3B and C). Interestingly, two distinct tumor cell populations were found within the tumors, one being characterized by a higher degree of anaplasia than the other, and the GCT apparently consisted of clonal expansions of both cell types (Figure 3D). Numerous areas of osseous metaplasia and cystic structures were also found in all tumors (Figure 3E and data not shown). Histopathological analyses of all tissues from *Pten*flox/flox;*Amhr2*cre/+ mice bearing GCT revealed the presence of nests of tumor cells in the lungs of all affected ani-mals. Although most of these consisted of tumor cell embolisms (i.e. still contained within a distended vascular structure), some were me-tastases, with clear invasion of the pulmonary parenchyma (Figure 3F and G). All emboli and metastases consisted exclusively of the more highly anaplastic cell type identified in the ovarian tumors, further indicating the more malignant phenotype of this cell type (Figure 3H). Occasional cystic areas, necrosis and osseous metaplasia were also observed within the emboli and metastases (data not shown). No tumor cells were detected in the lungs of *Pten*flox/flox;*Amhr2*cre/+ con-trol mice that had not developed ovarian tumors. These data demon-strate that *Pten* loss can lead to metastatic GCT development and therefore plays a central role in the pathogenesis of GCT.

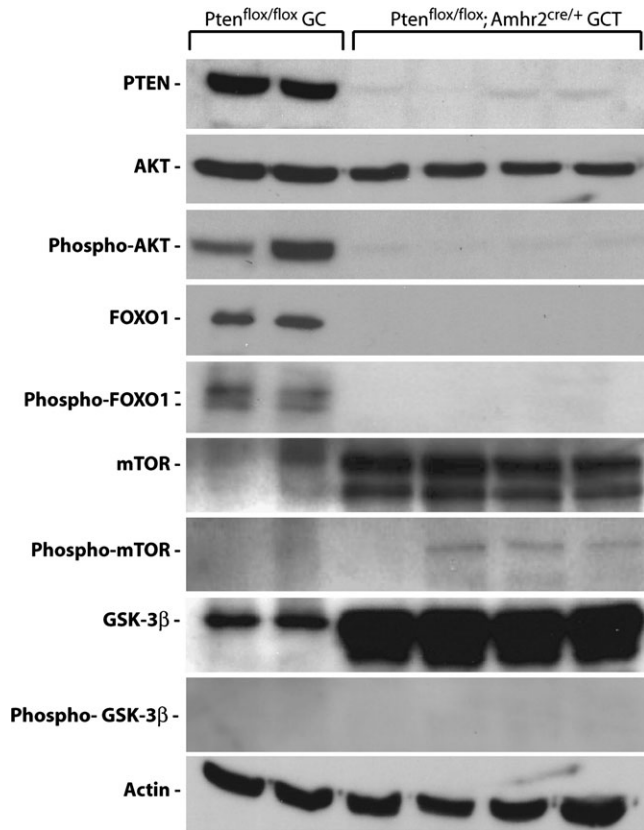### Altered PI3K/AKT signaling in Pten*flox/flox*;Amhr2*cre/+* GCT

To study the signaling mechanisms underlying GCT development *Pten*flox/flox;*Amhr2*cre/+ in mice, immunoblotting was performed com-paring the expression of PTEN and various PI3K/AKT pathway ef-fectors in *Pten*flox/flox;*Amhr2*cre/+ GCT to control granulosa cells from antral follicles. Unexpectedly, AKT phosphorylation in GCT was not increased, but rather was lower relative to granulosa cell controls, in spite of efficient recombination of the floxed *Pten* alleles (Figure 2B) and drastically decreased PTEN expression (Figure 4). Decreased phospho-AKT expression in *Pten*flox/flox;*Amhr2*cre/+ GCT was con-firmed by immunohistochemistry (Supplementary Figure 1, supple-mentary data are available at *Carcinogenesis* online), and phospho-AKT levels were comparable in the low and high anaplasia tumor cell types. Phosphorylation of the PI3K/AKT effector mTOR was detected at low levels in *Pten*flox/flox;*Amhr2*cre/+ GCT samples but not in gran-ulosa cells (Figure 4), suggestive of increased signaling via the PI3K/AKT/mTOR pathway. In addition, levels of the mTOR protein itself were also greatly increased in *Pten*flox/flox;*Amhr2*cre/+ GCT. Conversely, phosphorylation of GSK3β was essentially undetectable in either *Pten*flox/flox;*Amhr2*cre/+ GCT samples or in granulosa cells (Figure 4), suggesting that GSK3β is not a target of AKT in either cell type and that cross talk with the WNT/CTNNB1 pathway is not involved in tumorigenesis in the *Pten*flox/flox;*Amhr2*cre/+ model. Indeed, levels of GSK3β expression were considerably higher in *Pten*flox/flox;*Amhr2*cre/+ GCT than in granulosa cells, potentially result-ing in greater repression of WNT/CTNNB1 signaling. No increase in phospho-FOXO1 levels was found in *Pten*flox/flox;*Amhr2*cre/+ GCT relative to normal granulosa cells, and in fact phospho-FOXO1 was virtually undetectable by western blotting in all tumor samples ana-lyzed (Figure 4). This was attributed to a dramatic loss of FOXO1 expression in GCT (Figure 4). The proteosomal degradation of FOXO1 has been shown previously to occur in response to chronic activation of the PI3K/AKT pathway in transformed cells (54). Our results therefore suggest that loss of PTEN expression in *Pten*flox/flox; *Amhr2*cre/+ GCT alters PI3K/AKT pathway activity and that its signal may be transduced via both mTOR and FOXO1.
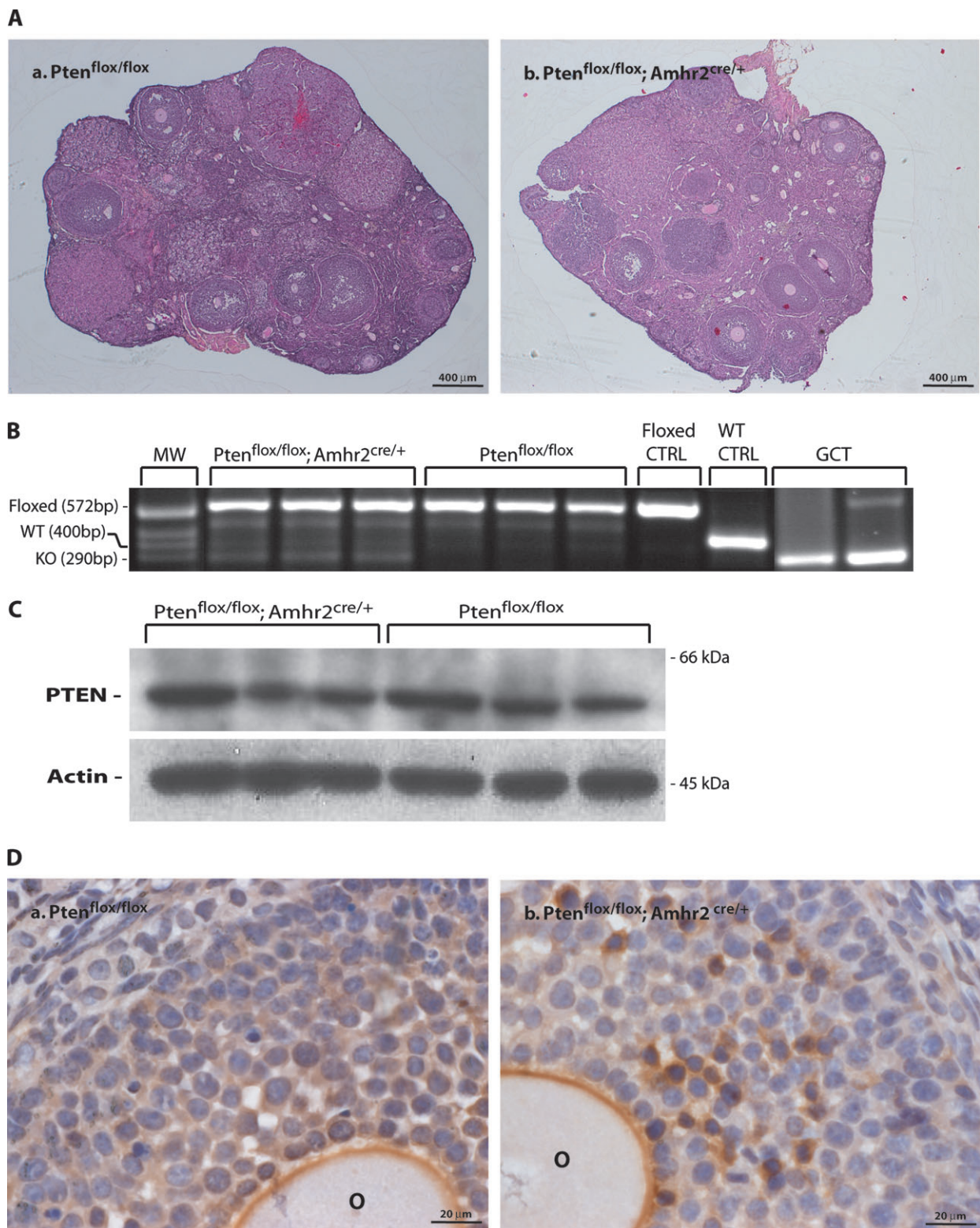
Fig. 2. Lack of ovarian anomalies in most *Pten*flox/flox;*Amhr2*cre/+ mice. (**A**) Histological sections of ovaries from 5-month-old *Pten*flox/flox and *Pten*flox/flox;*Amhr2*cre/+ mice. Original magnification, ×50. (**B**) PCR analysis of Cre-mediated recombination of the floxed *Pten* allele. Ovarian DNA was isolated from 1-month-old *Pten*flox/flox and *Pten*flox/flox;*Amhr2*cre/+ mice (second through seventh lanes, inclusively). Positive controls for the floxed allele (Floxed CTRL) and wild-type allele (WT CTRL) were DNA from tail biopsies taken from a *Pten*flox/flox and a wild-type mouse, respectively. Granulosa cells tumor DNA was isolated from 10-week-old *Pten*flox/flox;*Amhr2*cre/+ females (last two lanes). Expected PCR product sizes for the floxed, wild-type (WT) and cre-recombined (KO) alleles are indicated on the left. An additional ∼500 bp PCR product was commonly observed in samples of all genotypes and presumed to be an artifact unrelated to *Pten*. MW = molecular weight standards. (**C**) Immunoblot analyses of PTEN expression in isolated *Pten*flox/flox and *Pten*flox/flox;*Amhr2*cre/+ granulosa cells. Each lane represents a distinct protein sample derived from the pooled granulosa cells from three non-tumor bearing mice. Actin was used as a loading control. (**D**) Immunohistochemical analysis of phospho-AKT expression in *Pten*flox/flox and *Pten*flox/flox;*Amhr2*cre/+ ovaries. O = oocyte. Original magnification, ×1000.

Fig. 3. Rare *Pten*^{flox/flox};*Amhr2*^{cre/+} mice develop aggressive, metastatic GCT. (**A**) Abdominal cavity of a 10-week-old *Pten*^{flox/flox};*Amhr2*^{cre/+} mouse bearing two large GCT. (**B** and **C**) Microscopic views of solid (B) and trabecular (C) GCT histological patterns. Original magnifications, 100× (B) or 200× (C). (**D**) Photomicrograph showing distinct cell populations within *Pten*^{flox/flox};*Amhr2*^{cre/+} GCT, characterized by higher (HA) or lesser (LA) degrees of anaplasia. Original magnification, ×400. (**E**) Microscopic view of a focus of ossification (O) within a *Pten*^{flox/flox};*Amhr2*^{cre/+} GCT. Original magnification, ×200. (**F**) Photomicrograph showing the presence of tumor cell embolisms in the lungs of the animal shown in (A). The arrow indicates an example of an embolism (i.e. the

Fig. 4. Altered PI3K/AKT signaling in *Pten*$^{\text{flox/flox}}$;*Amhr2*$^{\text{cre/+}}$ GCT. Immunoblot analyses of the indicated PI3K/AKT pathway signaling effectors in *Pten*$^{\text{flox/flox}}$ granulosa cells (first two lanes) and *Pten*$^{\text{flox/flox}}$;*Amhr2*$^{\text{cre/+}}$ granulosa cells tumors (final four lanes). Actin was used as a loading control.

*Abnormal subcellular localization of phospho-AKT occurs in many human and equine GCT*

To assess whether dysregulated PI3K/AKT pathway activity occurs in spontaneously occurring GCT, a panel of human and equine GCT were analyzed for PTEN expression by immunohistochemistry. While PTEN abundance was very low in all GCT samples examined, similar results were also obtained in normal granulosa cells at all stages of follicular development (Figure 5B and data not shown), precluding any meaningful comparisons. It was therefore decided to evaluate *PTEN* expression by a more sensitive reverse transcription–PCR approach. *PTEN* mRNA was readily detectable in all human GCT samples examined ($n = 6$) and at levels comparable with those found in normal ovarian samples ($n = 5$) or isolated luteinizing granulosa cells (Figure 5C). We were thus unable to detect a gross loss of *PTEN* expression in our tumor samples. Immunohistochemical analyses of phospho-AKT expression in our GCT panel showed levels of phospho-AKT in all GCT samples to be qualitatively comparable with the levels found in granulosa cells in normal human and equine ovarian follicles (Figure 5A). However, the subcellular localization of phospho-AKT was often abnormal in the tumor samples. Specifically, two of five human GCT samples showed a striking perinuclear localization of phospho-AKT, while seven of 17 equine GCT samples showed markedly elevated levels of nuclear phospho-AKT expression (Figure 5A). Although the biological significance of nuclear localization of phospho-AKT remains unclear, it has been

reported in a variety of human cancers and may correlate in some cases with disease progression (27). These data therefore suggest that abnormal PI3K/AKT signaling occurs in many spontaneously occurring GCT and may be an important factor in human and equine GCT development.
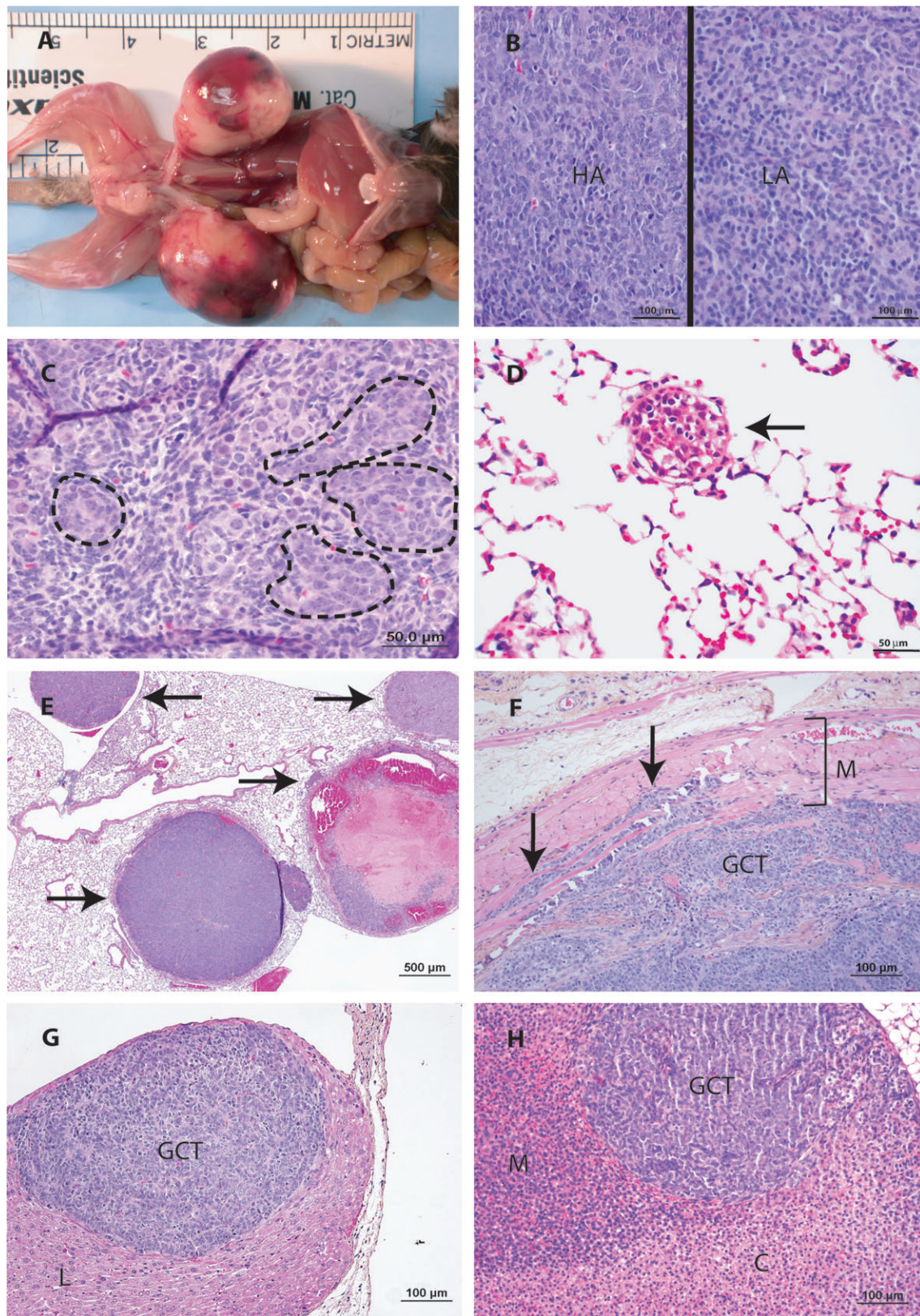
Pten$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ *mice develop early-onset metastatic GCT*

Interactions between the PI3K/AKT and WNT/CTNNB1 pathways have been reported in several forms of cancer (30,39–44). In addition, we have reported previously that WNT/CTNNB1 signaling is dysregulated in many GCT and that genetically engineered mice that feature constitutive activation of the WNT/CTNNB1 pathway in their granulosa cells (*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$) develop premalignant ovarian lesions that often evolve into GCT after the age of 5 months (5). We therefore decided to determine if the PI3K/AKT and WNT/CTNNB1 pathways could interact in GCT development. To this end, a mouse model (*Pten*$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$) was designed to obtain concurrent constitutive activation of both pathways in granulosa cells. These mice developed GCT similar to those observed in *Pten*$^{\text{flox/flox}}$;*Amhr2*$^{\text{cre/+}}$ mice at the gross and histological levels, including solid and trabecular histological patterns, variable degrees of anaplasia and foci of ossification (Figure 6A and B and data not shown). However, *Pten*$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ mice developed bilateral tumors with 100% penetrance from a very early age. Histopathological analyses revealed the presence of nests of dysplastic cells in the ovaries of newborn mice (Figure 6C) and e20.5 embryos, but not in those from mice on e18.5 (not shown), indicating that tumor growth began perinatally in *Pten*$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ mice. The disease then followed a very regular and predictable course (Supplementary Figure 2, supplementary data are available at *Carcinogenesis* online), with abdominal distension becoming evident by 5 weeks of age and severe by 7 weeks, with tumor diameters surpassing 2 cm. Death occurred before 9 weeks of age ($n = 3$), possibly due to severe anemia (as evidenced by low hematocrit and extensive extramedullary hematopoiesis), complicated by pressure from the tumors on the diaphragm and digestive tract and causing impaired venous return. *Pten*$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ mice also had pulmonary tumor cell embolisms (Figure 6D), but metastases were not observed, possibly due to insufficient time for the metastases to form due to the rapid and fatal course of the disease. To test this hypothesis, the GCT were surgically removed from five 6-week-old *Pten*$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ mice, and their lungs submitted for histopathological analysis 6–16 weeks postoperatively. All of these mice showed development of large lung metastases (Figure 6E), confirming the metastatic properties of *Pten*$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ tumor cells. In addition to forming pulmonary metastases, the more aggressive forms of human GCT also frequently spread by seeding of exfoliated tumor cells into the peritoneal cavity (2). To test if *Pten*$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ tumor cells could also behave in this manner, the GCT of five additional 6-week-old mice were removed, and cells (~ 1 mm^3) were scraped from the surface of the excised tumors, suspended in saline and injected into the peritoneal cavity following the closure of the abdominal wall. *Pten*$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ mice with injected tumor cells were then killed 6–9 weeks postoperatively and their tissues submitted for histopathological analyses. In addition to lung tumor cell emboli and metastases, these mice had multiple abdominal tumors invading the mesentery, peritoneum and abdominal muscles (Figure 6F and data not shown). The invasion and replacement of pancreatic tissue by tumor cells were also observed in one case, as were adrenal and liver metastases (Figure 6G and H and data not shown). These data therefore indicate that *Pten*$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ GCT

tumor cells are entirely contained within a vascular structure). Original magnification, ×200. (**G**) Photomicrograph showing the presence of GCT metastases (arrows) in the lungs of the animal shown in (A). Original magnification, ×400. (**H**) Photomicrographs comparing the highly anaplastic cells from the tumors (GCT) to those in the pulmonary metastases (PM). Original magnification, ×1000.
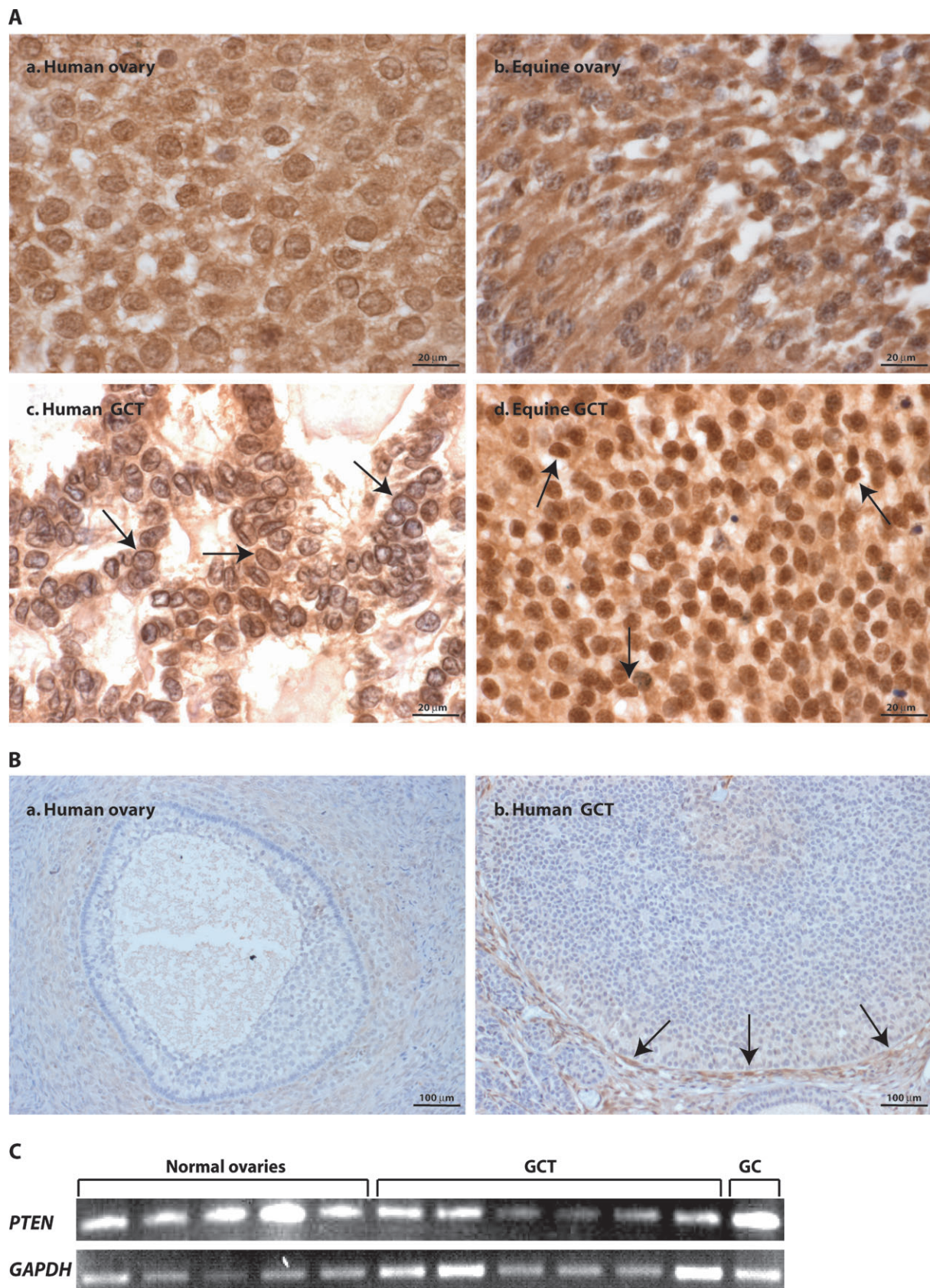
Fig. 5. Abnormal PI3K/AKT pathway activity occurs in many GCT. (**A**) Immunohistochemical analysis of phospho-AKT expression in human and equine normal ovaries and granulosa cells tumors. Arrows indicate examples of cells showing perinuclear (image c) or nuclear (image d) staining. Original magnification, ×1000. (**B**) Immunohistochemical analysis of PTEN expression in human normal ovaries and granulosa cells tumors. Original magnification, ×200. Arrows indicate staining in non-tumoral stromal cells, little or no staining was found in the tumor cells. (**C**) Semiquantitative reverse transcription–PCR analysis of *PTEN* expression in human normal ovaries, granulosa cells tumors and isolated luteinizing granulosa cells (GC). *GAPDH* was used as a control gene.

Fig. 6. *Pten*flox/flox;*Ctnnb1*flox(ex3)/+;*Amhr2*cre/+ mice develop early-onset GCT with a high penetrance. (**A**) A 6-week-old *Pten*flox/flox;*Ctnnb1*flox(ex3)/+;*Amhr2*cre/+ mouse bearing two large GCT. (**B**) Photomicrographs showing distinct cell populations within *Pten*flox/flox;*Ctnnb1*flox(ex3)/+;*Amhr2*cre/+ GCT, characterized by higher (HA) or lesser (LA) degrees of anaplasia. (**C**) Photomicrograph of a newborn *Pten*flox/flox;*Ctnnb1*flox(ex3)/+;*Amhr2*cre/+ ovary. Growing nests of dysplastic cells are circumscribed with dotted lines. Original magnification, ×400. (**D**) Photomicrograph of a pulmonary tumor cell embolism (arrow) from a 6-week-old female *Pten*flox/flox;*Ctnnb1*flox(ex3)/+;*Amhr2*cre/+ mouse. Original magnification, ×400. (**E–H**) Photomicrographs depicting GCT pulmonary metastases (panel E, arrows), GCT cell invasion of the abdominal wall (panel F, M = muscular layer of the abdominal wall, arrows indicate invading GCT cells), a liver GCT metastasis (panel G, L = liver) and an adrenal GCT metastasis (panel H, M = adrenal medulla, C = adrenal cortex) from *Pten*flox/flox;*Ctnnb1*flox(ex3)/+;*Amhr2*cre/+ mice killed several weeks after surgical removal of the primary ovarian tumors. Original magnification, ×40 (E) or ×200 (F–H).

cells are able to directly seed and colonize the pelvis and upper abdomen, in addition to forming pulmonary metastases.

Discussion

Meaningful insights into the molecular etiology of GCT have been frustratingly elusive. Not only are genes that are commonly mutated in many neoplasia apparently not involved in the pathogenesis of GCT (such as *TP53* and *WT1*), little evidence has been obtained of hyperactivation in GCT of signaling pathways normally involved in granulosa cell proliferation, despite many attempts (6–8). However, ongoing advances in our understanding of granulosa cell biology and the molecular mechanisms underlying their growth and differentiation are providing new avenues for the investigation of GCT development. For instance, the recent findings that WNT signaling is crucial for the normal embryonic development of the ovary (55,56) prompted our discovery that the WNT/CTNNB1 signaling pathway is dysregulated in many GCT and that constitutive activation of the WNT/CTNNB1 pathway in granulosa cells causes GCT development in *Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ mice (5,13). In a similar manner, the present study of the PI3K/AKT pathway in GCT was inspired by recent reports indicating that FSH signals via this pathway (22,57). Our finding that *Pten*$^{\text{flox/flox}}$;*Amhr2*$^{\text{cre/+}}$ mice develop GCT provides novel and powerful evidence that dysregulation of the PI3K/AKT pathway plays a major role in the pathogenesis of GCT. Furthermore, the metastatic phenotype observed in the *Pten*$^{\text{flox/flox}}$;*Amhr2*$^{\text{cre/+}}$ model is unique among the previously reported transgenic mouse models of GCT (5–8). Human GCT can metastasize to the lung and to bone (2), but the genetic and molecular mechanisms underlying the acquisition of the metastatic phenotype in these tumors has not been studied. The *Pten*$^{\text{flox/flox}}$;*Amhr2*$^{\text{cre/+}}$ model therefore provides the first insights into the signaling pathways involved in GCT progression.

Another important finding of this study is the synergistic interaction of the PI3K and WNT/CTNNB1 pathways in GCT development. Constitutive activation of the WNT/CTNNB1 pathway in the granulosa cells of *Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ mice results in a premalignant phenotype wherein follicle-like nests of disorganized granulosa cells appear in the ovaries of peripubertal mice, grow to the size of small antral follicles and then persist for the rest of the life of the animal (5,13). These premalignant lesions often develop into GCT, but only later in life, indicating that activation of the WNT/CTNNB1 pathway is a powerful initiator of granulosa cell tumorigenesis, but may be insufficient in and of itself to cause GCT to form. Conversely, the rarity of GCT formation in *Pten*$^{\text{flox/flox}}$;*Amhr2*$^{\text{cre/+}}$ mice in addition to the aggressive nature of their GCT phenotype suggest that activation of the PI3K/AKT pathway may be more involved in the progression of the GCT disease, but is rarely able to initiate it. The synergy between the PI3K/AKT and WNT/CTNNB1 pathways observed in the *Pten*$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ model could therefore be understood as a complementarity between pathways involved in tumor initiation (i.e. WNT/CTNNB1) and progression (i.e. PI3K/AKT). Importantly, the *Pten*$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ model features many of the morphological and functional characteristics of the most aggressive forms of human GCT disease, including lung metastasis tropism and the ability to spread by seeding into the peritoneal cavity. This suggests that the molecular mechanisms underlying *Pten*$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ tumorigenesis may be very similar to those involved in the advanced human disease. Particularly considering the predictable and repeatable nature of tumor development in *Pten*$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ mice, we therefore propose that they could serve as an important preclinical model for advanced GCT and could notably aid in the development of therapeutic interventions.

Loss of PTEN expression normally relieves an inhibition of PI3K activity, resulting in a sustained hyperphosphorylation of AKT in response to various stimuli. Our finding of decreased levels of AKT phosphorylation in *Pten*$^{\text{flox/flox}}$;*Amhr2*$^{\text{cre/+}}$ GCT relative to normal granulosa cells is therefore paradoxical. We propose that this may be due to the chronic nature of the activation of the PI3K/AKT pathway in our model, which could eventually lead to a cellular adaptive response that downregulates AKT phosphorylation, such as by increasing phosphatase activity. Indeed, the finding that FOXO1 expression is lost in *Pten*$^{\text{flox/flox}}$;*Amhr2*$^{\text{cre/+}}$ GCT supports this notion, as this is known to occur as a result of sustained PI3K/AKT pathway activation (54). FOXO1 is thought to act as a tumor suppressor in certain contexts, and loss of FOXO1 function may play a significant role in certain cancers such as alveolar rhabdomyosarcoma (58). FOXO1 has been implicated in many physiological processes, including the regulation of cell cycle progression and apoptosis (58). It therefore seems reasonable to propose that the loss of FOXO1 protein that we observed in *Pten*$^{\text{flox/flox}}$;*Amhr2*$^{\text{cre/+}}$ GCT could be a major mechanism by which GCT cells acquire an increased rate of proliferation and evade apoptosis. In addition to FOXO1, a large number of AKT substrates have been identified in several cell types, including BAD, caspase-9 and Mdm2 (59). Additional work will be required in order to determine if these or other effectors are involved in the pathogenesis of GCT in *Pten*$^{\text{flox/flox}}$;*Amhr2*$^{\text{cre/+}}$ mice.

The molecular mechanisms explaining the synergy between the PI3K and WNT/CTNNB1 pathways in *Pten*$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ mice remain unresolved. Activated AKT is well known to enhance WNT/CTNNB1 pathway activity by inhibiting GSK3β activity, resulting in the hypophosphorylation, stabilization and nuclear translocation of CTNNB1 (30,34–44) (Figure 1). However, in the *Ctnnb1*$^{\text{flox(ex3)}}$ model, WNT/CTNNB1 pathway activation is achieved by excision of the third exon of *Ctnnb1* by *Cre* recombinase (47). The recombined *Ctnnb1*$^{\text{flox(ex3)}}$ allele expresses a mutant CTNNB1 protein that, while still fully functional, lacks the N-terminal sites that are normally phosphorylated by GSK3β and that are required for its degradation (47). Therefore, in *Pten*$^{\text{flox/flox}}$;*Ctnnb1*$^{\text{flox(ex3)/+}}$; *Amhr2*$^{\text{cre/+}}$ mice, mutant CTNNB1 cannot be further activated by inhibition of GSK3β by AKT, indicating that a different mechanism must mediate the putative interaction between the PI3K and WNT/CTNNB1 pathways. One possibility is that AKT has recently been shown to directly enhance CTNNB1 transcriptional activity by phosphorylation at a distinct site (Ser 552), which is still present in the mutant CTNNB1 protein produced by the recombined *Ctnnb1*$^{\text{flox(ex3)}}$ allele (33). It is also possible that no direct cross talk exists between the PI3K and WNT/CTNNB1 pathways in *Pten*$^{\text{flox/flox}}$; *Ctnnb1*$^{\text{flox(ex3)/+}}$;*Amhr2*$^{\text{cre/+}}$ GCT and that the observed synergy in GCT development is simply the cumulative effect of each pathway's activation on its own discrete set of downstream effectors. Further experiments will be required to explore these non-mutually exclusive theories.

Our preliminary finding of abnormal subcellular localization of phospho-AKT in a subset of human and equine GCT suggests that dysregulation of PI3K/AKT signaling occurs in spontaneously occurring GCT. It remains to be determined if this dysregulation is a cause or a consequence of tumor development, what the causative genetic lesion(s) might be and what its relatedness may be to what occurs in the *Pten*$^{\text{flox/flox}}$;*Amhr2*$^{\text{cre/+}}$ model. We were unable to demonstrate loss of *PTEN* expression in human GCT; however, our analyses were limited by the relatively small sample size available, and that the technique that we employed could only detect gross loss of *PTEN* mRNA expression, providing no information on protein levels or function. Additional analyses will therefore be required to rule out *PTEN* loss as a mechanism for PI3K/AKT dysregulation in GCT. It should however be noted that other components of the PI3K/AKT pathway, including *AKT2* and both the regulatory and catalytic subunits of PI3K, are also frequently mutated or amplified in many human cancers (60). Furthermore, a growing number of oncogenic signaling processes are now known to activate the PI3K/AKT pathway, including mechanisms involving Ras, p53 and DJ1 (61,62), any or all of which could also contribute to dysregulation of PI3K/AKT activity in GCT. It therefore seems unlikely that simple loss of *PTEN* expression will explain all cases of dysregulated PI3K/AKT signaling in GCT, and considerable effort will be required to properly investigate this process.

The infertility phenotype observed in $Pten^{flox/flox}$;$Amhr2^{cre/+}$ mice remains an area of ongoing investigation. Although pregnancy loss could be attributable to a primary ovarian defect, our preliminary serum progesterone measurements suggest that corpus luteum function is unaffected in $Pten^{flox/flox}$;$Amhr2^{cre/+}$ mice. Furthermore, we have mated $Pten^{flox/flox}$ mice to a transgenic strain in which the *Cre* gene is fused to the FSH receptor promoter (*Fshr*-Cre) and that is thought to express Cre in granulosa cells exclusively (63). The resulting $Pten^{flox/flox}$;*TgFshr-Cre* mice have normal fertility and are apparently devoid of uterine defects (M.N.Laguë, L.Dubeau and D.Boerboom, unpublished observations), further suggesting that the fertility issues of $Pten^{flox/flox}$;$Amhr2^{cre/+}$ mice can be attributed to an extra-ovarian defect. Importantly, the $Amhr2^{cre}$ allele has recently been shown to target Cre-mediated recombination to the developing myometrium (64,65), indicating that loss of myometrial *Pten* expression could be the primary cause of pregnancy loss in $Pten^{flox/flox}$; $Amhr2^{cre/+}$. If true, the $Pten^{flox/flox}$;$Amhr2^{cre/+}$ model could therefore help define novel roles for *Pten* and PI3K/AKT signaling in uterine physiology and pregnancy.

In summary, this study reports for the first time the role of dysregulated PI3K/AKT signaling in GCT development, as well as evidence that the PI3K/AKT and WNT/CTNNB1 pathways can interact in a synergistic manner to cause granulosa cell tumorigenesis. The two novel animal models described herein, $Pten^{flox/flox}$;$Amhr2^{cre/+}$ and $Pten^{flox/flox}$; $Ctnnb1^{flox(ex3)/+}$;$Amhr2^{cre/+}$, represent the first models for metastatic GCT and provide the first true insights into the molecular mechanisms of GCT progression.

Supplementary material

Supplementary material can be found at http://carcin.oxfordjournals. org/.

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