Expression of Activated PIK3CA in Ovarian Surface Epithelium Results in Hyperplasia but Not Tumor Formation

Shun Liang¹, Nuo Yang¹, Yue Pan¹, Shan Deng¹, Xiaojun Yang¹, Dionyssios Katsaros⁴, Katherine F. Roby⁵, Thomas C. Hamilton⁶, Denise C. Connolly⁶, George Coukos¹,²,³, Lin Zhang¹,²,²⁺

¹ Center for Research on the Early Detection and Cure of Ovarian Cancer, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, United States of America, ² Department of Obstetrics and Gynecology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, United States of America, ³ Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, Philadelphia, United States of America, ⁴ Department of Obstetrics and Gynecology, University of Turin, Turin, Italy, ⁵ Center for Reproductive Sciences, University of Kansas Medical Center, Kansas City, Kansas, United States of America, ⁶ Ovarian Cancer Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania, United States of America

Abstract

Background: The Phosphatidylinositol 3'-kinase is a key regulator in various cancer-associated signal transduction pathways. Genetic alterations of its catalytic subunit alpha, PIK3CA, have been identified in ovarian cancer. Our in vivo data suggests that PIK3CA activation is one of the early genetic events in ovarian cancer. However, its role in malignant transformation of ovarian surface epithelium (OSE) is largely unclear.

Methodology/Principal Findings: Using the Müllerian inhibiting substance type II receptor (MISIIIR) promoter, we generated transgenic mice that expressed activated PIK3CA in the Müllerian epithelium. Overexpression of PIK3CA in OSE induced remarkable hyperplasia, but was not able to malignantly transform OSE in vivo. The consistent result was also observed in primary cultured OSEs. Although enforced expression of PIK3CA could not induce OSE anchorage-independent growth, it significantly increased anchorage-independent growth of OSE transformed by mutant K-ras.

Conclusions/Significance: While PIK3CA activation may not be able to initiate OSE transformation, we conclude that activation of PIK3CA may be an important molecular event contributing to the maintenance of OSE transformation initiated by oncogenes such as K-ras.

Introduction

Epithelial ovarian cancer continues to be the leading cause of death among gynecological malignancies [1,2,3]. The lack of effective methods for prevention, early detection and treatment recurrent ovarian tumors creates a pressing need to understand its pathogenesis and identify molecular targets for therapy. Cancer is a disease involving multistep dynamic changes in the genome [4]. However, the oncogenic events and their cooperation that promote malignant transformation in ovarian carcinoma remain largely unknown [2,3,5,6,7,8,9,10].

Phosphatidylinositol-3' kinase (PI-3 kinase) is an intracellular signal transducer with lipid substrate specificity implicated in a wide range of cancer-associated signaling pathways including tumor cell metabolism, survival and proliferation [11,12,13,14,15,16,17,18,19]. It is recruited and activated by multiple receptor tyrosine kinases and generates second messengers via phosphorylation of membrane inositol lipids at the D3 position [11,12,13,14]. PI-3 kinase was first recognized as a putative oncogene because of its ability to bind polypoma middle T antigen [20,21]. Molecular cloning of PI-3 kinases revealed a large and complex family that contains three classes with multiple subunits and isoforms [11,12,13,14]. The PIK3CA gene encodes the catalytic subunit p110-alpha, one of the three catalytic subunit proteins of the class IA PI-3 kinases [11,12,13,14]. PIK3CA was identified as an avian retrovirus-encoded oncogene that can transform chicken embryo fibroblasts [22]. Numerous recent studies indicate that PIK3CA and downstream pathways are frequently targeted by genomic amplification [23], mutation [24] or overexpression [25] in solid tumors including ovarian cancer [23,25,26,27,28,29,30,31,32]. Previous studies on the function of PIK3CA in ovarian cancers have been predominantly focused on the maintenance and survival of late-stage of ovarian carcinoma. The function of PIK3CA in malignant transformation of the ovarian surface epithelium (OSE) remains unexplored. Here we generated transgenic mice expressing constitutively activated (myr-isoIolated) PIK3CA in the Müllerian epithelium of the female genital tract to investigate the effect of PIK3CA overexpression in the OSE.
Results

**PIK3CA overexpression was an early genetic event during ovarian tumorigenesis**

In many human tumors, including epithelial ovarian cancer [23,25,26,27,28,29,30], **PIK3CA** activation is a critical oncogenic event and can be mediated by multiple genetic/genomic alterations such as gene copy number amplification [23], gain of functional mutations [24], and transcriptional up-regulation [33,34,35]. However, the function of **PIK3CA** activation during the process of malignant transformation of OSE is still not well understood. Our previous studies indicate that mRNA expression of **PIK3CA** is significantly up-regulated in the early-stage of ovarian cancer development, strongly suggesting that **PIK3CA** might be involved in OSE transformation [25]. To confirm our previous finding, we first compared expression levels of **PIK3CA** mRNA in established epithelial ovarian cancer cell lines (n = 15) with primary cultures of immortalized human ovarian surface epithelium (IOSEs, n = 6). Consistently, we found that mRNA levels in epithelial tumor cell lines were significantly higher than in IOSEs (p = 0.041, Figure 1A). Next, we examined mRNA expression of **PIK3CA** in microdissected normal human ovarian epithelium (n = 4) as well as epithelial ovarian cancer specimens including FIGO stages I (n = 16), II (n = 8), III (n = 31) and IV (n = 11). We found that mRNA expression level of **PIK3CA** was significantly upregulated in ovarian cancer specimens compared to normal control ovarian epithelium (p < 0.02), and there was no further significant increase after malignant transformation among different stages of ovarian cancer (p > 0.05, Figure 1B), which is consistent with our previous observation [25]. To further confirm **PIK3CA** is indeed expressed in early-stage ovarian cancer, we also examined the protein product of **PIK3CA** gene, p110α, by immunohistochemical staining in early malignant transformed human ovarian surface epithelium. We found that p110α was highly detectable in the early malignant transformed human ovarian surface epithelium (Figure 2A and B). Taken together,
these results indicate that PIK3CA overexpression is in fact an early genetic event during ovarian oncogenesis, thus suggesting that PIK3CA activation might be causally involved in this process.

Generation of transgenic mice expressing activated PIK3CA in the Mullerian epithelium

To investigate the role of PIK3CA in malignant transformation of the OSE in vivo, we generated transgenic mice in which activated PIK3CA was specifically overexpressed in the Mullerian epithelium of the female reproductive tract including OSE. In this model, we used the Mullerian epithelium specific promoter, Mullerian inhibiting substance type II receptor promoter (MISIR) [36,37,38,39], to drive expression of the murine PIK3CA (Figure 3). In the male animal, Mullerian inhibiting substance (MIS) is secreted from Sertoli cells of the developing testes and stimulates the regression of the Mullerian duct. Testosterone is also secreted from the developing testes and induces the differentiation of the Wolfian duct into the secondary structures of the male reproductive tract. In the absence of MIS in the developing female embryo, the Mullerian duct differentiates into the secondary structures of the female reproductive tract [36,37,38,39]. Expression of the MISIR has been reported to be restricted to mesenchymal cells surrounding the Mullerian duct during embryogenesis, tubular and follicular structures of fetal gonads, Sertoli and Leydig cells of adult testis, and granulosa cells of adult ovary [36,37,38,39]. Above information provides a possible strategy to develop a transgenic model of ovarian carcinoma [39]. Using this promoter, Connolly et al. have successfully developed the first ovarian cancer transgenic models that develop ovarian carcinomas with metastatic spread to peritoneal organs [39]. In addition, increasing evidence indicates that PIK3CA is activated in a large percentage of human ovarian cancer patients [25,27,33]. Therefore, we generated the activating mutation by addition of the avian src myristoylation sequence (MGSSSKP) at the N-terminus of the wild type of murine PIK3CA to constitutively activate PI3-kinase pathway in vivo. To demonstrate that our transgenic construct was able to constitutively activate PI3-kinase pathway, we transiently transfected myr-PIK3CA, wt-PIK3CA and control vectors to ovarian cancer cell line 2008. 48 hrs of post-transfection, the transfected cells were cultured in low serum overnight. Protein and total RNA were isolated from cells. Real-time RT-PCR demonstrated that cells from wt-PIK3CA and myr-PIK3CA transfections were expressed similar levels of PIK3CA mRNA, which was about 11.5-fold higher comparing to cells from control vector transfection. Total and phosphate AKT, the downstream molecule of PI3-kinase pathway, were examined by western blot. Figure 4 showed that myr-PIK3CA was able to constitutively activate AKT in low serum condition (1%) compared with wt-PIK3CA and control transfection.

Twelve founder lines of MISIR-PIK3CA transgenic (PIK3CA-Tg) mice were generated, in which the genomic integration of whole MISIRpr-myrPIK3CA-IRES-GFP sequence was confirmed by two sets of genotyping primers (Figure 5A). Expression of the transgene mRNA (eGFP) was examined by RT-PCR in the samples from the female whole ovary of the genotype-positive mice. In three of the transgenic lines, the eGFP mRNA transcript was able to be detected in vivo (Figure 5B). Three founder PIK3CA-Tg lines (#6, #22 and #26), three control lines (genotype positive, eGFP mRNA negative #3, #12 and #4, Figure 5B) and wild type mice (genotype negative mice) were used for further studies.

Overexpression of activated PIK3CA in OSE resulted in hyperplasia but not tumor formation

To confirm overexpression of PIK3CA in the OSEs of transgenic mouse, we microdissected the OSE cells from PIK3CA-Tg and WT mice using laser capture microdissection (LCM, Figure 6A). Total RNA was isolated, and PIK3CA mRNA expression was measured by real-time RT-PCR. We found that PIK3CA mRNA expression level was 18.5-fold higher in OSEs from PIK3CA-Tg mice (200.93 ± 39.47 relative expression unit) than from WT mice (10.87 ± 7.28 relative expression unit, p = 0.009, Figure 6B). In addition, by immunohistochemistry (using two different antibodies), we confirmed that p110α was highly expressed in the OSE of the PIK3CA-Tg mice (data not shown). Then, we followed up the ovarian tumor development in PIK3CA-Tg mice. We found that expression of the activated PIK3CA resulted in hyperplasia in mouse OSE and a paucity of follicles in four month-old female mice. Female transgenic PIK3CA-Tg mice exhibited subfertility. Figure 7A shows the typical OSE in 5-month control female mice. Female transgenic PIK3CA-Tg mice exhibited subfertility. Figure 7A shows the typical OSE in 5-month control female mice. PLoS ONE | www.plosone.org 3 January 2009 | Volume 4 | Issue 1 | e4295

Figure 3. The illustration of the construct that was used to generate transgenic mouse. The arrows show two sets of the genotyping primers and one set of the RT-PCR primers. doi:10.1371/journal.pone.0004295.g003

Figure 4. myr-PIK3CA was able to active PI3-kinase pathway. myr-PIK3CA, wt-PIK3CA and control vectors were transiently transfected to ovarian cancer cell line 2008. 48 hrs of post-transfection, transfected cells were cultured in 1% serum overnight. Total and phosphate AKT were detected by western blot. doi:10.1371/journal.pone.0004295.g004
on the surface of ovary as a monolayer. In the PIK3CA-Tg mice, hyperplasia was found in the OSE, and the epithelial origin of the lesions was confirmed by Cytokeratin staining (Figure 7B). Hyperplasia was found in more than 50% of the PIK3CA-Tg mice after four months post-birth, and in 100% of the PIK3CA-Tg mice after ten months post-birth. There were no invaginations or papillary structures observed in the OSE of PIK3CA-Tg mice. In the control mice, no significant hyperplasia in OSE was observed even after 12 months of post-birth. These results suggest that expression of activated PIK3CA in OSE induced hyperplasia of the OSE. We monitored for ovarian tumor development in both the transgenic and control mouse lines. A total of 218 female PIK3CA-Tg mice (#6: n = 94; #22: n = 44 and #26: n = 80) were evaluated (at least 30 mice of each line were followed for more than 18 months) and no epithelial ovarian tumors were observed. There was no difference in life span between the transgenic mice

Figure 5. Generation of transgenic mice expressing activated PIK3CA in the Müllerian epithelium. A. A total of 12 founder lines were generated. Upper panel shows the genotyping by PCR using the first set of the genotyping primers. Lower panel shows the genotyping by PCR using the second set of the genotyping primers. B. The transgene (eGFP) was able to be detected in 3 founder lines by RT-PCR. doi:10.1371/journal.pone.0004295.g005

Figure 6. PIK3CA was overexpressed in OSE of PIK3CA-Tg mouse. A. Ovarian surface epithelial cells were microdissected by laser capture microdissection technology. B. PIK3CA mRNA expression in OSEs from WT or PIK3CA-Tg OSEs was analyzed by real-time RT-PCR. The primers were able to detected both wt PIK3CA cDNA and cDNA from transgene expression (myr-PIK3CA). doi:10.1371/journal.pone.0004295.g006
PIK3CA ly and selected again by puromycin for 3 days. Interestingly, we
10 days G418 selection, pathways have been reported involving in OSE transformation, we
Because PI3-kinase/PTEN-ras [8] and PI3-kinase/AKT-myc [5]
(pUSEamp-myr-m
rasv12 and c-myc (both in pBabe-puro vector, puromycin)
activation of
PIK3CA c-myc
did not significantly increase colony numbers of OSE cells
compared with mutant
PIK3CA increased anchorage-independent growth of cultured OSE cells
co-transfection. The myr-
PIK3CA
expression vectors were transfected to MOSE cells. The pooled
stable expression cells were generated by short-term antibiotic
selection (10 days for G418 or 3 days for puromycin). The
transgene expression was further confirmed by RT-PCR. We
found that overexpression of PIK3CA alone did not significantly
increase anchorage-independent growth of OSEs (Figure 8A). In
contrast, introduce of either mutant K-ras or c-myc resulted in
increased colony formation of OSEs (Figure 8A). This result
indicates that unlike mutant K-ras or c-myc, PIK3CA is not able to
cause complete transformation of OSE in vitro. Next, we tested the
combination of PIK3CA with either of the two other oncogenes by
co-transfection. The myr-PIK3CA was transfected first. After
10 days G418 selection, K-ras or c-myc was selected subse-
cuently and selected again by puromycin for 3 days. Interestingly, we
found that combining PIK3CA and mutant K-ras significantly
increased anchorage-independent growth of cultured OSE cells
compared with mutant K-ras alone (Figure 8A). However, PIK3CA
did not significantly increase colony numbers of OSE cells
transformed by c-myc (Figure 8A). This finding suggests that early
activation of PIK3CA might promote transformation of OSE cells
in certain cellular and molecular contexts, e.g., in the presence of
K-ras mutation. To further confirm that PIK3CA can play a role in
transformation induced by mutant K-ras, we blocked endogenous
PIK3CA expression in cultured OSE cells transfected with mutant
K-ras by RNA interference using small interfering RNAs (siRNA).
The efficiency of the siRNA targeting mPIK3CA was confirmed by
real-time RT-PCR (mRNA expression of mPIK3CA was knocked
down to ~30% in the siRNA treated cells compared with control
cells, data not shown). The specificity of the siRNA was also
examined by measuring endogenous mPIK3CB expression (there was
no significantly difference of PIK3CB mRNA expression between
siRNA treated and control cells, data not shown). We
found that in the absence of PIK3CA, the transformed OSEs could
not grow in soft agar (Figure 8B), which suggests that the growth of
K-ras transfected OSE cells require PIK3CA expression. However,
it is still unclear whether PIK3CA contributes to K-ras initiated
transformation in vitro. Generation of “bigenic” mouse expressing
both activated PIK3CA and mutant Ras Specifically in murine OSE
using MISIR promoter is still a technical challenge. Because
mutant Ras is able to fully transform epithelium in vivo [42],
MISIR-driven mutant Ras will induce very rapidly reproductive
system carcinoma in both female and male animals, and these
general mice will lose reproductive ability. Therefore, it will be
difficult to generate “bigenic” animals by crossing MISIR-driven
mutant Ras and MISIR-driven myr-PIK3CA mice. We believe that
a novel transgenic strategy based on the Cre-loxp conditional
epression and intrabursal administration of adenovirus [6,8,9]
may allow us to further test PIK3CA and Ras cooperation in vivo in
the future.

Discussion
In agreement with our findings, several groups have demon-
strated that activation of PI3-kinase plays a crucial role in
epithelial cell transformation. For example, in human mammary
epithelial cells (HMECs), activation of the PI3-kinase pathway in
the presence of increased expression of c-myc, can functional
replace small T antigen and result in anchorage-independent

Figure 7. Overexpression of activated PIK3CA in OSE resulted in hyperplasia but not tumor formation. A. The ovarian surface epithelium in wild type mouse. B. The ovarian surface epithelium in PIK3CA-Tg mouse. The epithelium origin was confirmed by cytokeratin staining.
doi:10.1371/journal.pone.0004295.g007
growth [43]. In p53 null murine OSE cells, enforced expression of the PI3-kinase downstream mediator, Akt, in cooperation with mutant K-ras, induces the OSE cell transformation [5]. Moreover, two independent laboratories have recently reported that conditional Pten deletion combined with mutant K-ras [8] or deregulated Wnt/Catenin pathway [9] is able to induce endometrioid ovarian tumors in mice in vivo. In all of these studies, only activation of wild type PIK3CA alone seems insufficient to initiate epithelial transformation, consistent with the results of our study. Although mutant PIK3CA has been reported to be sufficient to transform normal cells both in vitro [44,45,46] and in vivo [46,47], PIK3CA mutation exhibits a relatively low frequency in ovarian cancer (4.8–12%) [28,29,30,32] as compared to other cancer types. In addition, PIK3CA mutation is rare in borderline [30] or early-stage ovarian tumors (our unpublished observation). Therefore, PIK3CA mutation might not be commonly involved in the transformation of the OSE. However, PIK3CA amplification is one of most common genetic alterations in ovarian cancer (23.6–40%) [23,26,27,31] and more importantly, an increase in copy number at chromosomal locus 3q26-qter, which harbors the PIK3CA gene, has been observed in 40% of early-stage ovarian cancers [26], suggesting that PIK3CA amplification might be one of the critical events in OSE transformation. In this study, we tested overexpression of activated wild type PIK3CA in OSE in vivo and in vitro, since it might more closely resemble natural OSE transformation during human ovarian cancer development. Taken together, we conclude that PIK3CA activation is one of the early molecular events during OSE transformation, and activation of PIK3CA contributes to tumorigenesis in certain cellular and molecular contexts. However, PIK3CA activation might not be the initial event in OSE transformation, and may require cooperation with other oncogenic events such as K-ras mutation to maintain transformed OSE growth.

Materials and Methods

Patients and Specimens

The specimens used in this study were collected at the University of Pennsylvania and the University of Turin, Italy. All tumors were from primary sites, and were immediately snap-frozen and stored at −80°C. Ethical approval for this work was granted by institution’s Institutional Review Boards (IRBs) of the University of Pennsylvania and the University of Turin. Tissues were obtained after informed written consent from patients involved under a general tissue collection protocol approved by the IRBs.

Cell lines and Cell Culture

A total of 15 ovarian cell lines were used in this study. All cancer cell lines were cultured in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen). Six independent immortalized human ovarian surface epithelial cells (IOSEs, generously provided by Drs. Auersperg and Birrer) were cultured in 1:1 media 199: MCDB 105 (Sigma, St. Louis, MO) supplemented with 15% FBS. Murine ovarian surface epithelial cells (MOSEs) were isolated and cultured as previously reported [40].

Plasmid Construction

pMISIRprom-TOPO, which contains the murine Mullerian inhibitory substance type II receptor (MISIIR) gene 5’ regulatory region, was constructed by PCR amplification from mouse genomic DNA [39]. pUSEamp-myr-mPIK3CA, which contains murine PIK3CA under the control of CMV promoter, was ordered from UPSTATE (UPSTATE, Lake Placid, NY). The activating mutation was generated by addition of the avian src myristoylation sequence (MGSSKSKPK) at the N-terminus. pCI-neo, which contains a chimeric intron, was ordered from Promega (Promega, Madison, WI). pMigR, which contains an internal ribosome entry site (IRES) and downstream enhanced green fluorescent protein (eGFP), was generously provided by Dr. Pear. We tagged myr-PIK3CA in pUSEamp with eGFP, preceded by an IRES. The IRES and eGFP were derived from pMigR. The XhoI-SalI-blunted fragment containing the IRES and eGFP from plasmid pMigR was inserted into the XhoI-AlaI-blunted plasmid pUSEamp-myr-PIK3CA to construct plasmid pUSEamp-myr-PIK3CA-IRES-GFP. The SacI-XhoI linearized fragment containing the chimeric intron

Figure 8. PIK3CA contributed to K-ras initiated transformation in vitro. A and B. Summary of colony numbers of the different combination of oncogenes in soft agar assay. doi:10.1371/journal.pone.0004295.g008
from plasmid PCI-neo was inserted into the *EcoRV*-*XhoI* linearized plasmid pMISIRprom-TOPO to construct plasmid pMISIR-prom-Intron. The *MluI*-*SalI* linearized fragment containing the *MISIR* promoter and the intron from plasmid pMISIRprom-Intron was swapped into plasmid pUSEamp-myr-*PIK3CA*-IRES-GFP to make plasmid pMISIR promot-Intron- myr-*PIK3CA*-IRES-GFP, in which the CMV promoter has been replaced by the *MISIR* promoter and the intron upstream of the *PIK3CA* ORF, as well as the IRES, eGFP reporter and hGH polyA downstream (for the detailed information for construction also see Figure S1). This construct was used to generate a transgenic mouse expressing myr-*PIK3CA* in the OSE.

### Protein Isolation and Western blot

Cultured cells were lysed in 200 µl of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% Triton X-100. Protein was separated by 10% SDS-PAGE under denaturing conditions and transferred to nitrocellulose membrane. Membranes were incubated with an anti-total AKT or anti-pAKT antibodies (1:1,000, Cell Signaling Technology, Danvers, MA), followed by incubation in anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:10,000; Cell Signaling Technology). Immunoreactive proteins were visualized using enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).

### Transgenic Mice

The animal study protocol was reviewed and approved by the institutional animal care and use committee (IACUC) of the University of Pennsylvania. Transgenic mice were generated by the University of Pennsylvania’s Transgenic & Chimeric Mouse Facility. The linearized transgene DNA fragment (*MISIR*-myr-*PIK3CA*-IRES-GFP) was injected by microinjection into pronuclei of day-0.5 embryos of the first generation of a hybrid genetic background of C57BL/6 and C3H (B6C3F1) mice. Injected embryos were implanted into the oviducts of day-0.5 pseudo pregnant female Swiss Webster mice.

### Genotyping

Tails from the resulting pups were clipped 3 weeks after birth for genotype analysis. Genomic DNA was isolated by the DNeasy Tissue kit (QIAGEN, Valencia, CA). Presence of the transgene was confirmed by PCR amplification of a 486-bp fragment (Frag1) of the intron-myrr-*PIK3CA* as well as a 324-bp fragment (Frag2) of the eGFP. Specific primers for eGFP were as follows: Frag1 forward primer: 5’-AGG CAC TCG GCA GGT AAT TAG TAT, Frag1 reverse primer: 5’-CAT GTT TGA TGG TGA CGA GTG TGC, Frag2 forward primer: 5’-CGA CAA CCA CTA CCT GAG CA, Frag2 reverse primer: 5’-TTA GGA AAG GAC AGT GGG AGT G. Genomic DNA (2 µl) was amplified in 25 µl of the PCR reaction containing 200 µmol/L each dNTP, 20 pmol of each primer, the standard buffer supplemented with 1.5 U *Taq* polymerase (Roche, Indianapolis, IN), and 1.5 mmol/L MgCl₂. After initial denaturation at 94°C for 4 minutes, 35 cycles of PCR were performed with denaturation at 94°C for 15 seconds, annealing at 55°C for 20 seconds, and extension at 72°C for 45 seconds. The last extension was at 72°C for 7 minutes. Specific primers for GFP were as follows: eGFP forward primer: 5’-AGC TGA CCC TGA AGT TCA TCT G, eGFP reverse primer: 5’-GAT CTT GAA GTT CAC CTT GTA GGC.

### Real-time RT-PCR

cDNA was quantified by real-time RT-PCR on the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR was performed using Sybr Green PCR Core reagents (Applied Biosystems). PCR amplification of the housekeeping genes GAPDH was performed for each sample as a control for sample loading and to allow normalization among samples. A standard curve was constructed containing the *PIK3CA* cDNA and amplified by the real-time PCR. Each sample was run in duplicate and each PCR experiment included two non-template control wells.

### Laser capture microdissection (LCM)

LCM was performed as described by our previous study [48]. Briefly, cryosections (10 µm) from human or mouse ovaries were mounted on a polyethylene foil slide (SL Microtest, Jena, Germany). After rapid hematoxylin staining, sections were subjected to LCM utilizing the µCUT Laser-MicroBeam System (SL Microtest) with a fine ultraviolet laser, which enables the contact-free isolation of single cells or groups of cells. The microdissected cells were catapulted into the lid of a 0.5-ml reaction tube containing RNA isolation buffer. RNA was isolated by TRIzol reagent.

### Immunostaining

Immunohistochemical staining was performed using the avidin-biotin-peroxidase method [49]. Sections were pretreated with 0.03% H₂O₂ for 20 minutes to block endogenous peroxidase activity and incubated in matched normal sera (Vector Laboratories, Burlingame, CA). The following primary antibodies were used in this study: Cytokeratin (Hybridoma Bank, Iowa City, IA, 1:200) rabbit anti-p110α (Cell Signaling, Danvers, MA 1:200) and mouse anti-human p110α (Pharmigen, CA, 1:200). Mouse on Mouse (M.O.M.) kit (Vector) was used for monoclonal antibody. The Vectastain ABC kit was applied as described by the manufacturer (Vector Laboratories). Sections were counterstained with Gill’s hematoxylin (Vector Laboratories). Images were acquired through a Cool SNAP Pro color digital camera (Media Cybernetics).

### RNA interference/Transfection of Synthetic siRNA

Synthetic SMARTpool siRNAs targeting mouse *PIK3CA* (Dharmacon, Chicago, IL) or appropriate siCONTROL non-targeting siRNAs (Dharmacon) were transfected into cultured cells. Transfection was performed using Lipofectamine™2000 (Invitrogen) following the manufacturer’s instructions. Forty-eight hrs post transfection, total RNA was extracted to examine the *PIK3CA* expression by real-time RT-PCR.

### In vitro Cell Transformation Assay

A soft agar assay was performed using Cell Transformation Detection Assay Kit (Chemicon, Temecula, CA) following the manufacturer’s instructions [41].
mouse generation; Drs. Steven Johnson and Kang-Shen Yao (University of Pennsylvania) for the human ovarian cancer cells; Dr. Michael J. Birrer (NCI) for HOSE cells; Dr. Nelly Auerberg (University of British Columbia) for HOSE cells and access to the Canadian Ovarian Tissue Bank; Dr. Pear (University of Pennsylvania) for pMigR vector; and Saundra Ehrlich for editing assistance on this manuscript.

**Author Contributions**

Conceived and designed the experiments: GC LZ. Performed the experiments: LS NY YP SD XL XY LZ. Analyzed the data: LS LZ. Contributed reagents/materials/analysis tools: DK KR TH DC. Wrote the paper: GC LZ.

**References**

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, et al. (2007) Cancer statistics, 2007. CA Cancer J Clin 57: 43–66.
2. Auerberg N, Wong AS, Choi KC, Kang SK, Leung PC (2001) Ovarian surface epithelium: biology, endocrinology, and pathology. Endo Rev 22: 255–288.
3. Landen CN Jr, Birrer MJ, Sood AK (2008) Early events in the pathogenesis of epithelial ovarian cancer. J Clin Oncol 26: 995–1005.
4. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100: 57–70.
5. Orucul S, Li Y, Slosor RA, Vitale-Cross LA, Gutkind JS, et al. (2002) Induction of ovarian cancer by defined multiple genetic changes in a mouse model system. Cancer Cell 1: 53–62.
6. Flecken-Nikitin A, Choi KC, Eng JP, Shimizu EN, Nikitin AY (2003) Induction of carcinogenesis by concurrent inactivation of p53 and RB1 in the mouse ovarian surface epithelium. Cancer Res 63: 3459–3463.
7. Liu J, Yang G, Thompson-لانza JA, Glassman A, Hayes K, et al. (2004) A genetically defined model for human ovarian cancer. Cancer Res 64: 1655–1663.
8. Diniuscu DM, Ince TA, Quade BJ, Shafer SA, Crowley D, et al. (2005) Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer. Nat Med 11: 63–70.
9. Wu R, Hendrix-Lucas N, Kruik R, Zhai Y, Schwartz DR, et al. (2007) Mouse model of human ovarian endometrioid adenocarcinoma based on somatic defects in the Wnt-beta-catenin and PI3K/Pten signalling pathways. Cancer Cell 11: 321–333.
10. Chodankar R, Kwan S, Sangjori F, Hong H, Yen HY, et al. (2005) Cell-nonautonomous induction of ovarian and uterine serous cystadenomas in mice lacking a functional Brcal in ovarian granulosa cells. Curr Biol 15: 561–565.
11. Bader AG, Kang S, Zhao L, Vogt PK (2005) Oncogenic PI3K deregulates transcription and translation. Nat Rev Cancer 5: 921–929.
12. Vivanco I, Sawyers CL (2002) The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer 2: 489–501.
13. Hemmety BT, Smith DL, Pati PT, Costello E, Mills GB (2005) Exploiting the PI3K/ AKT pathway for cancer drug discovery. Nat Rev Drug Discov 4: 985–1004.
14. Engleman JA, Lu Y, Canley LC (2006) The evolution of phosphotidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev. Genet 7: 609–619.
15. Jia S, Liu Z, Zhang S, Liu P, Zhang L, et al. (2008) Essential roles of PI3K/ p110beta in cell growth, metabolism and tumorigenesis. Nature.
16. Parsons R (2005) Phosphotyrosylkinase 3-kinase inhibitors are a triple threat to ovarian cancer. Clin Cancer Res 11: 7965–7966.
17. Hu L, Hofmann J, Lu Y, Mills GB, Jaffe RB (2002) Inhibition of phosphotidylinositol 3-kinase increases efficacy of paclitaxel in vitro and in vivo ovarian cancer models. Cancer Res 62: 1067–1092.
18. Hu L, Hofmann J, Jaffe RB (2005) Phosphotyrosylkinase 3-kinase mediates angiogenesis and vascular permeability associated with ovarian carcinoma. Clin Cancer Res 11: 8208–8212.
19. Sazl LH, Johansson F, Hohn K, Groener-Sazl SK, She QB, et al. (2007) Poor prognosis in ovarian cancer is associated with a gene expression signature of aberrant PTEN tumor suppressor pathway activity. Proc Natl Acad Sci U S A 104: 7564–7569.
20. Sugimoto Y, Whitman M, Canley LC, Erikson RL (1986) Evidence that the Rous sarcoma virus transforming gene product phosphorylates phosphotidylinositol and diacylglycerol. Proc Natl Acad Sci U S A 101: 2117–2121.
21. Whitman M, Kaplan DR, Schallhausen B, Canley I, Roberts TM (1985) Association of phosphotyrosylkinase kinase activity with polyoma middle-1 transforming protein. Nature 315: 293–297.
22. Chang HW, Aoki M, Fruman DA, Auger KR, Bellacona A, et al. (1997) Transformation of chicken cells by the gene encoding the catalytic subunit of PI-3 kinase. Science 276: 1048–1050.
23. Shavesteh L, Lu Y, Kuo WL, Baldocchi R, Godfrey T, et al. (1999) PIK3CA is implicated as an oncogene in ovarian cancer. Nat Genet 21: 99–102.
24. Samuels Y, Wang Z, Bordelli A, Silliman N, Pukh J, et al. (2004) High frequency of mutations of the PIK3CA gene in human cancers. Science 304: 554.
45. Zhao JJ, Cheng H, Jia S, Wang L, Gjoerup OV, et al. (2006) The p110alpha isoform of PI3K is essential for proper growth factor signaling and oncogenic transformation. Proc Natl Acad Sci U S A 103: 16296–16300.

46. Isakoff SJ, Engelman JA, Irie HY, Luo J, Brachmann SM, et al. (2005) Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells. Cancer Res 65: 10992–11000.

47. Bader AG, Kang S, Vogt PK (2006) Cancer-specific mutations in PIK3CA are oncogenic in vivo. Proc Natl Acad Sci U S A 103: 1475–1479.

48. Zhang L, Yang N, Park JW, Katsaros D, Fracchioli S, et al. (2005) Tumor-derived vascular endothelial growth factor up-regulates angiopoietin-2 in host endothelium and destabilizes host vasculature, supporting angiogenesis in ovarian cancer. Cancer Res 65: 3403–3412.

49. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massohrio M, et al. (2003) Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 348: 203–213.