PKC\(\text{\textsc{i}}\) Maintains a Tumor-initiating Cell Phenotype That Is Required for Ovarian Tumorigenesis

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Abstract

Protein kinase \(\text{\textsc{i}}\) (PKC\(\text{\textsc{i}}\)) has oncogenic potential and is an attractive therapeutic target for treatment of lung cancer, particularly those tumors that express elevated PKC\(\text{\textsc{i}}\). However, whether PKC\(\text{\textsc{i}}\) is a viable target in ovarian cancer is unknown, and virtually nothing is known about the mechanism by which PKC\(\text{\textsc{i}}\) drives ovarian tumorigenesis. Here, it is demonstrated that PKC\(\text{\textsc{i}}\) maintains a tumor-initiating cell (TIC) phenotype that drives ovarian tumorigenesis. A highly tumorigenic population of cells from human ovarian cancer cell lines exhibit cancer stem-like TIC properties, including self-renewal, clonal expansion, expression of stem-related genes, enhanced transformed growth \textit{in vitro}, and aggressive tumor-initiating activity \textit{in vivo}. Genetic disruption of PKC\(\text{\textsc{i}}\) inhibits the proliferation, clonal expansion, anchorage-independent growth, and enhanced tumorigenic properties of ovarian TICs. Biochemical analysis demonstrates that PKC\(\text{\textsc{i}}\) acts through its oncogenic partner Ect2 to activate a MEK/ERK signaling axis that drives the ovarian TIC phenotype. Genomic analysis reveals that PKC\(\text{\textsc{i}}\) and Ect2 are coordinately amplified and overexpressed in the majority of primary ovarian serous tumors, and these tumors exhibit evidence of an active PKC\(\text{\textsc{i}}\)-Ect2 signaling axis \textit{in vivo}. Finally, this study reveals that aurano\(\text{\textsc{n}}\), a potent and selective inhibitor of oncogenic PKC\(\text{\textsc{i}}\) signaling, inhibits the tumorigenic properties of ovarian TIC cells \textit{in vitro} and \textit{in vivo}. These data demonstrate that PKC\(\text{\textsc{i}}\) is required for a TIC phenotype in ovarian cancer, and that aurano\(\text{\textsc{n}}\) is an attractive therapeutic option to target deadly ovarian TICs in ovarian cancer patients.

Implications: PKC\(\text{\textsc{i}}\) drives a tumor-initiating cell phenotype in ovarian cancer cells that can be therapeutically targeted with aurano\(\text{\textsc{n}}\), a small molecule inhibitor of PKC\(\text{\textsc{i}}\) signaling. Mol Cancer Res; 11(12); 1624–35. ©2013 AACR.

Introduction

Ovarian cancer is the fifth leading cause of cancer-related death in women in the United States owing largely to late diagnosis and a high relapse rate after initial response to conventional therapy. As a result of this clinical course, the overall 5-year survival rate of ovarian cancer is only 15% to 30% (1). Clinical relapse is thought to be due to the survival and regrowth of highly tumorigenic ovarian cancer stem-like or tumor-initiating cells (TIC) after chemotherapy.

TICs are a relatively rare subpopulation of cells within the bulk tumor mass that has the ability to self-renew and give rise to the heterogeneous cancer cell lineages that comprise the tumor. The characteristic properties of TICs include increased lifespan, self-renewal, expression of stem-related genes, clonal expansion, enhanced transformed growth, resistance to chemotherapy, and the ability to efficiently initiate tumors in immune-compromised mice. These properties allow TICs to survive conventional platinum- and/or taxane-based therapy and cause relapse. However, the cellular mechanisms underlying the oncogenic behavior associated with ovarian TICs are still poorly understood.

Protein kinase \(\text{\textsc{i}}\) (PKC\(\text{\textsc{i}}\)), a member of the protein kinase \(\text{\textsc{c}}\) family, was found to be a target for frequent tumor-specific gene amplification in multiple human cancer types, including cancers of the lung, stomach, head and neck, colon, breast, and ovary (2–9). In these tumor types, PKC\(\text{\textsc{i}}\) gene amplification drives PKC\(\text{\textsc{i}}\) expression, which in turn is associated with poor prognosis. PKC\(\text{\textsc{i}}\) confers resistance to chemotherapy-induced apoptosis of human leukemia cells, and inhibition of PKC\(\text{\textsc{i}}\) expression or activity sensitizes chronic myelogenous leukemia cells to chemotherapeutic agent-induced apoptosis (10). Furthermore, overexpression of kinase-deficient PKC\(\text{\textsc{i}}\) or knockdown of PKC\(\text{\textsc{i}}\) expression blocked anchorage-independent growth and invasion of human non–small cell lung cancer (NSCLC) cells and human pancreatic ductal adenocarcinoma (PDAC) cells (2, 11, 12), showing that PKC\(\text{\textsc{i}}\) is also required for maintenance of the transformed phenotype of cancer cells. In addition, expression of constitutively active PKC\(\text{\textsc{i}}\) in the intestinal epithelium led to an increase in carcinogen-induced colon tumors, whereas
expression of kinase-deficient PKC\(\text{z}\) had the opposite effect (4), demonstrating that PKC\(\text{z}\) promotes intestinal tumorigenesis in vivo.

In the lung, PKC\(\text{z}\) forms an oncogenic complex with its binding partners Par6 and the Rhô family guanine nucleotide exchange factor, Ect2 (13, 14). The formation of this complex activates a downstream signaling axis consisting of Rac1, Pak, Mek, and Erk, which drives oncogenic growth and tumor formation in NSCLC and PDAC cells (2, 13). On the basis of a genomic analysis of NSCLC, matrix metalloproteinase 10 (MMP10; stromelysin 2) was identified as a transcriptional target and downstream effector of the oncogenic PKC\(\text{z}\) signaling axis (11). Interestingly, MMP10 was found to promote the expansion of Kras-mediated bronchioalveolar stem cells (BASC) and to be required for BASC maintenance, tumor initiation, and metastatic potential in a murine model of Kras-mediated lung adenocarcinoma (15, 16). Taken together, these results indicate a strong functional link between PKC\(\text{z}\) and oncogenic TIC behaviors. On the basis of these results, we hypothesized that PKC\(\text{z}\) may drive cancer formation through the maintenance of a TIC phenotype in human tumors.

We recently identified the anti-rheumatic gold compounds, aurothiomalate and aurothioglucose, as small-molecule inhibitors of oncogenic PKC\(\text{z}\) that can inhibit oncogenic PKC\(\text{z}\) signaling in lung cancer cells (14, 17). These compounds function by interacting with a critical cysteine residue within the PKC\(\text{z}\) PB1 domain thereby disrupting binding of Par6 to PKC\(\text{z}\), leading to inhibition of oncogenic PKC\(\text{z}\) signaling (14). Our preclinical studies in the lung indicate that tumor PKC\(\text{z}\) expression is a critical determinant of response to treatment with these gold-containing compounds (17). In the present study, we demonstrate for the first time that PKC\(\text{z}\) plays a pivotal role in promoting the oncogenic behavior of ovarian TICs, including clonal expansion, enhanced transformed growth, and tumor initiation in vivo. We also demonstrate for the first time that auranofin, a gold compound in the same chemical class as aurothiomalate and aurothioglucose that is still available for clinical use, is an effective inhibitor of PKC\(\text{z}\) signaling and the ovarian TIC phenotype. Our data provide a compelling rationale for an ongoing clinical trial to assess dosing and initial efficacy of auranofin for treatment of serum ovarian cancer patients in a maintenance setting.

Materials and Methods

Cell lines and antibodies

Human SKOV3 and mouse ID8 cells were provided by the tissue core resource of the Mayo Clinic Ovarian SPORE (directed by Dr. K. Knutson, Mayo Clinic, Rochester, MN). SKOV3 and ID8 cells were cultured in Dulbecco’s Modified Eagle Medium + 4.5 g/L D-glucose + 1-glutamine (Life technologies) supplemented with sodium pyruvate, nonessential amino acids (NEAA; Life technologies), and 10% FBS (Life technologies). ES2 cells were obtained from the American Type Culture Collection and cultured in McCoy’s 5A + 1-glutamine (Life technologies) supplemented with NEAA (Life technologies), 15 mmol/L HEPES, and 10% FBS. PKC\(\text{z}\) antibody was purchased from BD Biosciences, phospho-Mek, Mek, phospho-Erk, Erk, and glyceralddehyde—3-phosphate dehydrogenase antibodies were purchased from Cell Signaling Technology. FLAG antibody was purchased from Sigma-Aldrich. Phospho-T328-Ect2 (pECT2) antibody was produced and characterized as described previously (16). Horseradish peroxidase-conjugated secondary antibodies were purchased from KPL.

Enrichment and culture of ovarian cancer oncospheres

Oncospheres were enriched from SKOV3, ES2, and ID8 ovarian cancer cell lines by culturing adherent cells (50,000 cells/mL) in ultra-low attachment culture flasks (Sigma-Aldrich) with DMEM-F12 medium containing 50 μg insulin (Sigma-Aldrich), 0.4% albumin bovine fraction V (Sigma-Aldrich), N-2 plus media supplement (Life technologies), B-27 supplement (Life technologies), 20 μg/mL EGF (PeproTech), and 20 μg/mL fibroblast growth factor (PeproTech) for 4 weeks. Oncospheres were passaged by trypsinization and resuspension of single cells in non-adherent stem cell culture medium. For redifferentiation, oncospheres were collected and replated in adherent cell culture conditions (10,000 cells/mL) for 2 weeks using growth media containing 10% FBS as described above for each cell line.

Lentiviral RNAi constructs, cell transduction, and immunoblot analysis

A validated and characterized lentiviral construct containing RNA interference (RNAi) sequence against the 3’ untranslated region of human PKC\(\text{z}\) was obtained from the Sigma-Aldrich Mission short hairpin RNA (shRNA) library and packaged into lentiviruses as described previously (11). A lentiviral vector containing an shRNAi, which recognizes no human genes, was used as a nontarget control (NT-RNAi). RNAi infections were performed as described previously (11). At 48 hours postinfection, the infected cells were treated with 5 μg/mL of puromycin for selection of a stably transduced cell population.

Cellular proliferation, clonal expansion, and anchorage-independent growth assays

Cellular proliferation was assessed using the MTT reduction assay. In short, ovarian cancer cells were trypsinized and plated in 96-well cell culture plates (5,000 cells/well). Cells were collected and assayed for proliferation every 24 hours according to the standard protocol provided by the manufacturer (Promega). Clonal expansion of oncosphere cells was assessed by isolating single cells in 96-well ultra-low attachment plates by serial dilution (one cell/well). The expansion of single cells was monitored by phase-contrast microscopy over a 15-day period. Oncospheres containing more than 10 cells at day 15 were considered to have undergone expansion. Anchorage-independent growth of ovarian cancer cells grown in adherent culture (referred to as parental cells), and of oncosphere cells, was assayed as described previously (12). Cell colonies were fixed, stained with Giemsa (EMD...
Chemicals), and quantified using ImagePro Plus 7.0 (Media Cybernetics, Inc.). All experiments were independently repeated at least three times.

**Tumorigenicity in mice**

The ability of ovarian cancer cells to grow as orthotopic tumors was assessed in immune-deficient nude mice (Harlan-Sprague-Dawley). Briefly, 5- to 7-week-old female mice were anesthetized with ketamine/xylazine, a small midline abdominal incision was made, the left ovary was externalized, and 20 μL containing 1,000 viable ovarian cancer cells previously transfected with an expression plasmid containing firefly luciferase suspended in sterile PBS were orthotopically injected into the left ovary under a dissecting microscope. SKOV3 or ES2 cells were injected into immune-deficient nude mice, whereas ID8 cells, an ovarian cancer cell line derived from a C57 mouse, were injected into syngeneic C57 BL/6J mice. Tumor growth was monitored using the IVIS imaging system (Caliper Life Sciences). Final tumor dimensions were determined with calipers as described previously (2).

**Results**

**Ovarian cancer oncospheres exhibit a tumor-initiating cell phenotype**

Given the potential importance of TICs in ovarian tumorigenesis, we enriched for cells exhibiting a highly tumorigenic TIC phenotype by culturing ovarian cancer cells in defined serum-free stem cell medium under low-attachment conditions. Two human ovarian cancer cell lines (SKOV3 and ES2) were used for these experiments. Both cell lines readily formed oncospheres when placed in stem cell culture conditions (Fig. 1A). To investigate whether these cell cultures are enriched in stem-like cells, we assessed expression of a panel of stem cell markers in parental, oncosphere, and oncosphere cultures that were placed back into adherent culture (Fig. 1B). Interestingly, both ES2 and SKOV3 oncospheres exhibited elevated expression of MMP10, a gene that we previously demonstrated was a transcriptional target of PKCι signaling in lung cancer cells (11), and which we demonstrated is important for Kras-mediated lung tumor initiation in mice (19).

To investigate whether oncosphere cultures acquire aggressive oncogenic behavior associated with a stem-like phenotype, we performed soft agar assays to assess anchorage-independent growth of parental, oncosphere, and redifferentiated oncosphere cells (Fig. 1C). We found that oncospheres from both cell lines exhibited increased anchorage-independent growth when compared with control parental cultures from which they were derived (Fig. 1C). Interestingly, when oncospheres are placed back into adherent culture in the presence of serum-containing media, the cells lose their enhanced soft agar colony formation and resemble parental control cells (Fig. 1C). Finally, we assessed the ability of parental and oncosphere cells to initiate tumors in vivo. For this purpose, we developed an orthotopic ovarian tumor model in which ovarian tumor cells are injected orthotopically into the capsule of the ovary of immune-deficient nude mice. Initial experiments determined that a minimum of 100,000 parental SKOV3 or ES2 cells were required to establish tumors with 100% tumor take (data not shown). In contrast, injection of as few as 1,000 ES2 or SKOV3 oncosphere cells led to 100% tumor take and development of large tumors within the injected ovary (Fig. 1D), and injection of 100 ES2 or SKOV3 oncosphere cells yield a tumor take of 40% (2/5) and 20% (1/5), respectively; therefore, 1,000 cells were used as the inoculum size for subsequent studies. In contrast with oncosphere cells, 1,000 parental ES2 cells failed to form tumors, demonstrating the enhanced tumorigenic, tumor-initiating properties of ES2 oncosphere cells (Fig. 1D). Given the tumor-initiating properties of oncospheres, we will henceforth refer to these cells as TICs.

**Ovarian TICs require PKCι for clonal expansion and enhanced anchorage-independent growth**

Our finding that expression of MMP10, a gene that we previously identified as a transcriptional target and downstream effector of oncogenic PKCι signaling in the lung (11), was elevated suggested that PKCι signaling is activated in, and important for the maintenance of, TIC cultures. Consistent with this hypothesis, immunoblot analysis demonstrates that PKCι expression is higher in ES2 oncosphere cells when compared with parental cells, whereas that of the related PKCζ was not (Supplementary Fig. S1A). To directly assess the role of PKCι in ovarian TIC proliferation and transformed growth, we performed PKCι knockdown (PKCι KD) utilizing a previously characterized and validated lentiviral-based shRNA construct targeting PKCι (11). The efficiency of PKCι knockdown was confirmed by quantitative PCR (qPCR) and immunoblot analysis (Fig. 2A). We next examined whether PKCι is important for clonal expansion of the ovarian TICs. Single TICs (NT or PKCι KD) isolated from oncosphere cultures were plated as single cells in nonadherent culture in individual wells of 96-well plates
and their ability to clonally expand into oncospheres was followed over a 15-day period (Fig. 2B). Although NT TICs efficiently expanded into large oncospheres, the majority of PKC
 kd TICs from both ES2 and SKOV3 failed to expand with the majority of clones remaining as single cells (Fig. 2B). Quantitative analysis of individual clones demonstrated that although NT TICs exhibit a very high clonal expansion efficiency (93% for SKOV3 and 67% for ES2), PKC
 kd TICs from both cell lines exhibited a significant reduction in clonal expansion efficiency (13% for SKOV3 and 7% for ES2; Fig. 2C). Thus, PKC
 plays a critical role in clonal expansion of ovarian TICs in vitro.

To determine the role of PKC
 in the enhanced anchorage-independent growth exhibited by TIC cultures, we performed soft agar assays on NT and PKC
 KD TICs (black bars) and parental cells (white bars; Fig. 2D). PKC
 KD significantly inhibits soft agar colony formation in both SKOV3 (left) and ES2 (right) TICs and parental cells when compared with NT KD cells. The specificity of the effect of PKC
 KD was confirmed by expressing exogenous PKC
 in PKC
 KD cells, which significantly restored soft agar colony formation (Supplementary Fig. S1B).

Ovarian TICs exhibit PKC
-dependent tumor-initiating activity in vivo

To evaluate the effect of PKC
 on the tumor-initiating potential of TICs in vivo, we used the ovarian cancer orthotopic mouse model described in Materials and Methods. SKOV3 NT parental cells, NT TICs and PKC
 KD TICs (1,000 viable cells/injection), expressing firefly luciferase were injected into the ovary of immune-deficient nude mice, and tumor growth was monitored using IVIS imaging (Fig. 3A). At 60 days after injection, imaging revealed large tumors in mice injected with NT oncosphere cells (left) but either very small or no detectable tumor formation in mice injected with either PKC
 KD TICs or NT parental cells (right). The results of IVIS imaging over the 60-day period revealed that only mice injected with NT TICs formed large tumors.
tumors, whereas both PKC\(i\)KD TICs and NT parental cells failed to initiate and maintain tumors (Fig. 3B). Similar results were obtained when parental ES2 or ES2 TICs were injected (Fig. 3C). Thus, PKC\(i\) is required for the tumor-initiating potential of human ovarian cancer TICs in vivo.

PKC\(i\) plays a critical role in the murine ovarian tumor-initiating cell phenotype.

To determine whether PKC\(i\) also plays a role in the TIC phenotype of murine ovarian cancer, we assessed the role of PKC\(i\) in the behavior of TICs isolated from the murine ovarian tumor cell line ID8 (Fig. 4). ID8 was isolated from an ovarian carcinoma tumor in a C57BL/6J mouse. TIC cultures from ID8 cells were established by growing the cells in nonadherent conditions in defined serum-free stem cell medium as described in Materials and Methods. Similar to our results in human ovarian tumor cell lines, ID8 cells grew efficiently as oncospheres in nonadherent culture (Fig. 4A). ID8 oncospheres exhibited enhanced anchorage-independent growth that is lost upon redifferentiation of the oncosphere by returning them to adherent culture in serum-containing medium (Fig. 4B). Furthermore, efficient knockdown of PKC\(i\) (Fig. 4C) led to a profound inhibition of clonal expansion (Fig. 4D and E), anchorage-independent growth (Fig. 4F), and tumor-initiating activity of ID8 oncospheres in vivo (Fig. 4G and H). We conclude that PKC\(i\) is required for the maintenance of the TIC phenotype in mouse ID8 cells, indicating that PKC\(i\) plays a general role in ovarian tumorigenesis.

The PKC\(i\)-Ect2 signaling axis is activated in ovarian TICs and primary ovarian tumors

We previously demonstrated that oncogenic PKC\(i\) signaling in the lung requires interaction of PKC\(i\) with its binding partner Par6, and that PKC\(i\)-Par6 binding recruits the Rho family GTPase GEF Ect2 to the complex (11, 13). PKC\(i\) directly phosphorylates Ect2 at T328 (16). PKC\(i\)-mediated Ect2 phosphorylation regulates the ability of Ect2 to activate Rac1 (16), which in turn activates a Mek-Erk signaling cascade that regulates the expression of MMP10 in a PKC\(i\)-dependent fashion (Fig. 5A; refs. 11, 13). To assess whether this oncogenic PKC\(i\) signaling mechanism is operative in ovarian TICs, we assessed the effect of PKC\(i\) KD on the activity of key components of this signaling pathway (Fig. 5B). Immunoblot analysis of cellular extracts from NT and PKC\(i\) KD ES2 TICs demonstrated that PKC\(i\) KD had little or no effect on total Ect2 expression, but led to a significant loss of \(pEct2\) in PKC\(i\) KD TICs when compared...
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PKC\(i\) KD also led to a concomitant decrease in both Mek and Erk phosphorylation levels (Fig. 5B) and to a decrease in MMP10 mRNA expression (Fig. 5C). To assess the functional role of the PKC\(i\)-Par6-Ect2-Mek-Erk-MMP10 signaling axis in TIC behavior, we assessed the effect of RNAi-mediated KD of Ect2 and MMP10, key effectors of this pathway downstream of PKC\(i\), on TIC behavior (Supplementary Fig. S2). Ect2 KD in ES2 TICs led to a decrease in MMP10 expression, and both Ect2 and MMP10 KD led to a decrease in clonal expansion of ES2 TICs. Taken together, these data indicate that the oncogenic PKC\(i\)-Par6-Ect2-Mek-Erk-MMP10 signaling axis is active in ovarian TICs and is important for TIC behavior. Because of the atypical PKC subfamily consists of two related isoforms, PKC\(i\), and PKC\(z\), we assessed whether PKC\(z\) has a similar effect on ovarian TIC behavior and signaling. PKC\(z\) KD in ES2 oncosphere cells, using our previously characterized shRNA lentiviral constructs (11), had little or no effect on clonal expansion or MMP10 expression, indicating that PKC\(z\) does not play a major role in ovarian oncosphere behavior or PKC\(i\) signaling (Supplementary Fig. S3).

To assess whether the PKC\(i\) signaling pathway characterized above is relevant to primary ovarian tumors, we interrogated gene expression in a dataset consisting of 489 ovarian serous carcinoma cases within The Cancer Genome Atlas (TCGA). Analysis revealed that \textit{PRKCI} and \textit{ECT2} exhibit coordinate gene copy number gains in approximately 80% of ovarian serous tumors as part of the chromosome 3q26 amplicon (Fig. 5D). Furthermore, gene expression analysis demonstrated a statistically significant and positive correlation between \textit{PRKCI}, \textit{ECT2}, and MMP10 mRNA levels in ovarian serous tumors (Fig. 5E). Taken together, these data demonstrate that \textit{PRKCI} and \textit{ECT2} are genetically and biochemically linked in primary ovarian tumors, and suggest that in tumors harboring \textit{PRKCI} and \textit{ECT2} copy number gains, the PKC\(i\)-Par6-Ect2–Mek-Erk-MMP10 signaling axis is activated.

The PKC\(i\) inhibitor auranofin potently inhibits PKC\(i\) signaling and ovarian TIC behavior

We recently identified the anti-rheumatoid gold compounds, aurothiomalate and aurothioglucose, as potent and selective inhibitors of oncogenic PKC\(i\) signaling that act by inhibiting the interaction between PKC\(i\) and Par6, thereby disrupting the PKC\(i\)-Par6-Ect2 signaling complex (14, 20). Unfortunately, these compounds are no longer available for clinical use. Therefore, we assessed the efficacy of auranofin, a gold compound in the same chemical class, to inhibit PKC\(i\) signaling. Given the critical role of PKC\(i\) signaling in ovarian TIC behavior, we assessed the effects of auranofin on the oncogenic properties of ovarian TICs. Consistent with a role for the PKC\(i\)-Par6 complex in oncogenic PKC\(i\) signaling, we observed a dose-dependent inhibition of TIC proliferation in the presence of auranofin with an apparent IC\(_{50}\) of approximately 200 nmol/L (Fig. 6A). To assess whether the inhibitory effects of auranofin on TIC growth is associated with inhibition of PKC\(i\) signaling, we assessed the effect of auranofin on PKC\(i\) pathway intermediates (Fig. 6B and C).

**Figure 3.** PKC\(i\) is required for the tumor-initiating activity of ovarian TICs in vivo. A, \textit{in vivo} luminescence (IVIS) imaging of representative mice 60 days after injection orthotopically with 1,000 viable cells derived from NT parental SKOV3 cells, NT SKOV3 TICs, or PKC\(i\) KD SKOV3 TICs. TICs exhibit enhanced tumor-initiating ability compared with parental cells. PKC\(i\) KD inhibits the enhanced tumor-initiating properties of SKOV3 TICs. B, SKOV3 NT parental cells, NT TIC and PKC\(i\) KD TIC tumor growth, were monitored by IVIS at the indicated time points after injection of 1,000 viable cells into recipient mice. Data are expressed as mean of total luminescence flux \(_{\text{SEM}}\), \(n = 4\), \(P < 0.05\) compared with parental NT (P-NT) or PKC\(i\) KD TICs. C, orthotopic ES2 tumors were assessed for tumor size after injection of 1,000 ES2 NT parental, NT TICs and PKC\(i\) KD TICs, as described in Materials and Methods. Data are expressed as mean tumor size (mm\(^3\)) \(_{\text{SEM}}\), \(n = 6\), \(P < 0.05\) compared with NT TICs.
Expression of FLAG-Par6 in ovarian TICs allowed us to directly assess binding of endogenous PKC to Par6 in the presence and absence of aurano. Aurano treatment led to a loss of Par6-bound PKC as determined by FLAG immunoprecipitation and PKC immunoblot analysis (Fig. 6B, top). Immunoblot analysis further revealed that aurano treatment inhibited pEct2, pMek and pErk levels (Fig. 6B, bottom), and MMP10 expression (Fig. 6C) when compared with dimethyl sulfoxide (DMSO)-treated control cells. Furthermore, transient expression of an aurothiomolate-resistant PKC mutant, PKC-C69I (14), in ES2 TICs can inhibit aurano-induced loss of MMP10 (Supplementary Fig. S4). Taken together, these data demonstrate that aurano inhibits the oncogenic PKC-Par6-Ect2-Mek-Erk-MMP10 signaling axis in ovarian TICs.

Given the ability of aurano to inhibit PKC signaling in ovarian TICs, we next assessed the effect of aurano on the tumorigenic behavior of TICs. Treatment of ES2 TICs with aurano led to significant inhibition of clonal expansion of TICs (Fig. 6D). This inhibitory effect manifested itself in both a dose-dependent decrease in the number of individual TICs that clonally expand (Fig. 6E) and in the size of oncospheres in the presence of aurano (Fig. 6F). Aurano treatment also led to a significant decrease in expression of stem cell markers (Fig. 6G) and anchorage-independent growth of TICs in soft agar (Fig. 6H). Taken together, these data demonstrate that pharmacologic inhibition of PKC with aurano blocks the TIC phenotype in vitro.

To assess the ability of aurano to inhibit ovarian TIC-mediated tumor formation in vivo, we established orthotopic ES2 TIC tumors in immune-deficient nude mice and treated tumor-bearing mice with aurano as described in Materials and Methods. As expected, injection of 1,000 ES2 TICs led to efficient establishment of tumors that were detectable by...
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Figure 5. PKC\textsubscript{i} activates a PKC\textsubscript{i}-Par6-Ect2-Mek-Erk signaling cascade in ovarian TICs. A, schematic of the oncogenic PKC\textsubscript{i} signaling cascade identified in NSCLC cells. B, PKC\textsubscript{i} KD in ES2 TICs causes a decrease in phosphorylation of T328 on Ect2, a previously characterized PKC\textsubscript{i} phosphorylation site on Ect2, and commensurate decreases in pMek and pErk levels. C, PKC\textsubscript{i} KD inhibits expression of MMP10, a transcriptional target of PKC\textsubscript{i}. Data are expressed as mean MMP10 RNA abundance normalized to NT control, n = 3, * P < 0.05 compared with NT control. D, PKC\textsubscript{i} and ECT2 are coamplified in primary ovarian serous carcinomas. Oncoprint readout of PRKCI and ECT2 gene copy number gains in primary ovarian serous carcinomas from TCGA dataset. Data reveal that PRKCI and ECT2 are virtually always coamplified in approximately 80% ovarian serous carcinoma tumors. E, analysis of PRKCI, ECT2, and MMP10 mRNA expression in primary ovarian serous tumors from the TCGA dataset reveal a strong positive correlation between expression of these three genes.

bioluminescence imaging by day 11, at which time tumor-bearing mice were randomly placed into one of two treatment groups that received either auranofin or diluent. Mice in the auranofin treatment group exhibited significant inhibition of tumor growth when compared with diluent-treated animals (Fig. 7A). By day 27 after tumor injection, diluent-treated mice exhibited signs of morbidity that necessitated termination of the experiment, whereas auranofin-treated mice showed no signs of morbidity. Analysis of tumor tissue from diluent and auranofin-treated mice revealed a decrease in mitotic index of tumor cells from auranofin-treated mice when compared with diluent control mice (Fig. 7B). Biochemical analysis revealed that auranofin treatment led to a significant decrease in tumor pERK levels (Fig. 7C) and pEct2 staining (Fig. 7D and E) consistent with inhibition of the PKC\textsubscript{i}-Ect2-Mek-Erk signaling axis identified in TICs in vitro. Thus, auranofin is a potent PKC\textsubscript{i} inhibitor that blocks ovarian TIC behavior in vitro and tumor formation in vivo.

Discussion

Ovarian cancer is the fifth leading cause of cancer death in women. A major contributing factor to the poor prognosis of patients with ovarian cancer is the high relapse rate of tumors after initial response to platinum-based chemotherapy with resistant disease. Recent studies have indicated that relapse and acquisition of chemoresistance is likely due to the survival and subsequent outgrowth of a relatively rare population of highly malignant cells within the primary tumor termed cancer stem cells or TICs. Ovarian TICs are characterized by elevated expression of stem cell marker genes and the ability to grow as oncospheres in nonadherent culture in stem cell media. TICs also exhibit an increased ability to clonally expand, grow as colonies in soft agar, and initiate tumors in vivo (21). In addition, ovarian TICs possess resistance to chemotherapeutic agents such as cisplatin providing further support for their involvement in relapse (22, 23). On the basis of these considerations, TICs have been hypothesized to be a critical tumor cell population that must be therapeutically targeted to elicit lasting tumor responses and improved patient survival.

In the present study, we cultured adherent SKOV3 and ES2 human ovarian cancer cells, and ID8 murine ovarian cancer cells, in defined serum-free stem cell media under low attachment conditions to select for stem-like cell populations. Each of the cell lines were able to readily form oncospheres enriched in cells exhibiting characteristic features of TICs, including clonal expansion, expression of stem marker genes, enhanced anchorage-independent growth, and efficient tumor-initiating activity when injected orthotopically into the ovary of recipient mice. An interesting aspect of our findings is that SKOV3 and ES2 TICs, while both expressing elevated stem-associated markers, expressed a distinct pattern of such markers, suggesting that these cell lines are maintained by distinct stem-like cell populations. These differences in stem cell marker gene expression patterns among oncosphere populations suggest the heterogeneous nature of ovarian cancer cells, and underscore the difficulty of identifying TICs based solely on expression of specific stem cell marker genes. Despite these differences, we did identify several genes whose expression was elevated in TICs.
from both cell lines, including ALDH1, CD44, and MMP10. The finding that MMP10, which encodes the MMP10, is elevated in ovarian TICs, including in murine ID8 TICs (data not shown), is significant because we had previously identified MMP10 as a critical transcriptional target of PKC\(\alpha\) in the lung (11). This observation led us to assess the role of PKC\(\alpha\) in maintenance of the ovarian TIC phenotype.

We previously demonstrated that PKC\(\alpha\) is required for tumor formation in the LSL-Kras model of lung adenocarcinoma (24). In this mouse model, the initiating step in tumor formation seems to be the activation of oncogenic
Kras in, and the subsequent transformation and expansion of, BASCs. Thus, BASCs are thought to serve as TICs in this mouse model; our findings demonstrated that PKCi is essential for Kras-mediated transformation of BASCs, and suggested that PKCi may play a similar role in the maintenance of TICs in fully transformed human tumor cells. Our present results demonsrate that genetic disruption of PKCi leads to a significant inhibition of the TIC phenotype in both human and murine ovarian TICs. Our results indicate that oncogenic PKCi signaling represents an intrinsic mechanism by which ovarian TICs maintain their tumorigenic, stem-like properties.

In the lung, we identified an oncogenic PKCi-Par6-Ect2-Mek-Erk-MMP10 signaling axis that is required for the ability of PKCi to support transformed growth of lung cancer cells in vitro and in vivo (11–13). Our current studies are consistent with a key role of this signaling pathway in PKCi-dependent maintenance of an ovarian TIC phenotype; PKCi KD leads to loss of phosphorylated Ect2 (a direct PKCi substrate), pMek and pErk, as well as expression of MMP10. Furthermore, treatment of ovarian TICs with the selective PKCi inhibitor auranoﬁn, which inhibits PKCi signaling through disruption of the PKCi-Par6 oncogenic complex, leads to similar inhibition of the PKCi-Par6-Ect2-

**Figure 7.** Auranofin (ANF) inhibits PKCi signaling and ovarian tumor growth in vivo. Orthotopic ES2 TIC tumors were established in immune-deﬁcient nude mice by orthotopic injection of 1,000 ES2 TICs into the capsule of the ovary as described in Materials and Methods. At day 11, tumor-bearing mice were randomly assigned to receive either auranoﬁn (12 mg/kg/d/6 days a week) or the same volume and frequency of vehicle solution (NaCl, 0.9%) for the duration of the experiment. A, quantitative analysis of tumor growth by IVIS bioluminescence. The results are expressed as mean fold-change of luminescence in each treated mouse compared with day 11 ±SEM, n = 5; *, P < 0.05. B, sections from tumors were analyzed for mitotic index as described in Materials and Methods. Tumors from auranoﬁn-treated mice exhibited a decrease in mitotic index compared with diluent-treated control mice, n = 5; *, P < 0.05. C, immunoblot analysis (top) of tumor lysates from diluent- and auranoﬁn-treated mice revealed a decrease in pErk levels in auranoﬁn-treated tumors. Quantitative analysis of pErk blots demonstrates a significant decrease in pErk levels in auranoﬁn-treated mouse tumors when compared with diluent-treated control mice, n = 3; *, P < 0.05. D, immunohistochemical staining of representative diluent- and auranoﬁn-treated tumors for pEct2. Auranofin treatment led to a decrease in pEct2 staining when compared with diluent control tumors. Staining was abolished by preincubation with Ect2 phospho-peptide antigen as described previously (16) indicating the specificity of the staining for pEct2 antigen. E, quantitative analysis of pEct2 immunohistochemical staining reveals a significant decrease in pEct2 staining in auranoﬁn-treated tumors compared with diluent control tumors, n = 5; *, P < 0.05.
Mek-Erk-MMP10 signaling cascade and potent inhibition of oncogenic TIC behavior. Our data further demonstrate that oncogenic PKCι signaling is active in ovarian TICs in vivo and that either genetic or pharmacologic inhibition of PKCι leads to a significant blockade of tumor initiation in vivo.

We previously demonstrated that PKCι is overexpressed in primary NSCLC cell lines and primary tumors, and PKCι expression is associated with poor clinical outcome in patients with NSCLC (3). In lung squamous cell carcinomas, PKCι expression is driven by frequent chromosome 3q26 amplification (3). Similar findings have been reported in ovarian serous tumors (7, 8), and PKCι has been shown to be important for the transformed phenotype of ovarian cancer cell lines in vitro (7). However, little was known about the molecular mechanisms by which PKCι drives the oncogenic phenotype in ovarian cancer cells. We have validated and extended these published findings, showing that approximately 80% of ovarian serous tumors in the TCGA dataset exhibit gene copy number gains in PRKCI, which is associated with elevated PRKCI mRNA expression. In the lung, PKCι is genetically, biochemically, and functionally linked to the Rho family GTPase GEF Ect2 (13). Our current findings demonstrate that PRKCI and ECT2 are coamplified and overexpressed in ovarian serous tumors as part of a 3q26 amplicon, and that PKCι and Ect2 are functionally linked to drive a PKCι-Par6-Ect2-Mek-Erk signaling pathway that is active in ovarian TICs. Analysis of the TCGA primary ovarian primary tumor data further demonstrate a strong positive correlation between the tumor expression of PKCι, Ect2, and MMP10 suggesting that the PKCι-Par6-Ect2-Mek-Erk-MMP10 signaling axis identified in ovarian TICs in vitro is activated in primary ovarian serous tumors. Taken together, our data indicate that PKCι promotes a PKCι-Par6-Ect2-Mek-Erk-MMP10 signaling axis that drives an ovarian TIC phenotype in vitro and in vivo. Furthermore, our data provide compelling evidence that PRKCI and ECT2 are genetically, biochemically, and functionally linked in primary ovarian serous tumors. These data suggest a novel paradigm in which a single genetic event, chromosome 3q26 amplification, leads to coordinate amplification and overexpression of two cooperating oncogenes that together drive an ovarian TIC phenotype. Our findings further demonstrate that the selective PKCι inhibitor auranocept can disrupt the TIC phenotype in vitro and in vivo. These data provide a compelling rationale of the use of auranocept for treatment of patients with ovarian cancer, particularly ovarian serous tumors harboring PRKCI and ECT2 gene copy number gains and activation of oncogenic PKCι signaling. We have initiated a phase I clinical trial to assess the feasibility of using auranocept in patients with ovarian serous cancer at high risk for relapse after initial therapy, a process likely driven by ovarian TICs that survive initial therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: Y. Wang, A.P. Fields
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Wang, K.S. Hill, A.P. Fields
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References

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