SHORT COMMUNICATION

PIK3CG Is a Potential Therapeutic Target in Androgen Receptor—Indifferent Metastatic Prostate Cancer

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The prostate epithelium consists of predominantly luminal cells that express androgen receptor and require androgens for growth. As a consequence, the depletion of testicular androgens in patients with prostate cancer results in tumor regression. However, it eventually leads to a castration-resistant disease that is highly metastatic. In this report, a mouse model of metastatic prostate cancer was generated through the deletion of the tumor-suppressor gene *Trp53* in conjunction with oncogenic activation of the proto-oncogene *Kras*. These mice developed early-onset metastatic prostate cancer with complete penetrance. Tumors from these mice were poorly differentiated adenocarcinoma, characterized by extensive epithelial–mesenchymal transition. With no or a very low level of androgen receptor expression, the tumor cells were resistant to androgen receptor inhibition. *Pik3cg*, encoding phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit γ (Pi3k γ), was highly expressed in these tumors, and pharmacologic inhibition of Pi3k γ blocked tumor cell growth *in vitro*, reversed epithelial–mesenchymal transition, and abated tumor metastasis *in vivo*. Immunohistochemistry analysis in human prostate cancer specimens showed that the expression of PIK3CG was significantly associated with advanced clinical stages. Taken together, these results suggest that PIK3CG plays an important role in the progression and metastasis of prostate cancer, and may represent a new therapeutic target in the metastatic castration-resistant prostate cancer. (*Am J Pathol* 2020, 190: 2194–2202; <https://doi.org/10.1016/j.ajpath.2020.07.013>)

The prostate is a hormone-dependent gland where androgen hormones bind to androgen receptor (AR) in the prostate epithelial cells, activating a signaling cascade that is crucial for the proliferation and differentiation of these cells. AR-mediated signaling also promotes the survival and proliferation of prostate cancer cells. As a result, androgen-deprivation therapy is the first-line treatment for prostate cancer. However, advanced prostate cancers eventually progress to a hormone-refractory state termed castration-resistant prostate cancer (CRPC), in which AR signaling is often reactivated through a variety of mechanisms, including AR gene mutations, amplification and overexpression, ligand-independent AR variants, and intratumoral and adrenal androgen synthesis. In recent years, second-generation AR inhibitors including abiraterone, apalutamide, darolutamide, and enzalutamide have been used

for further repressing AR signaling with improved efficacy in the treatment of CRPC. Although these drugs extend survival, they rarely lead to a complete remission. An emerging mechanism of resistance to AR-targeted therapy involves epithelial plasticity, in which tumor cells exhibit low to absent AR expression and demonstrate AR-independent growth.^{1,2} In fact, the loss of AR expression and/or downstream signaling occurs in an estimated 15% to 20% of castration-resistant tumors.³ Chemotherapy using docetaxel and cabazitaxel among others has been reserved as a treatment option for patients with metastatic CRPC, but acquired resistance to these drugs

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eventually also leads to fatality.⁴ Since treatment options at this stage are limited, the identification of novel therapeutic targets is needed for the treatment of advanced prostate cancer.

In advanced cancers, there are cells with a variety of different genetic alterations that are believed to contribute to disease aggressiveness and drug resistance. Among the most common genomic aberrations, the deletion or mutation of *PTEN* has been identified in approximately 20% of primary prostate tumors and in as many as 50% of CRPC. Loss of phosphatase and tensin homologue (PTEN) function is principally involved in the activation of the phosphoinositide 3-kinase (PI3K) pathway and has been associated with adverse outcomes.⁵ It was recently reported that gain-of-function mutations in *PIK3CA* (causing increased p110 α activity) and PTEN loss coexist in prostate cancer and cooperate to accelerate tumorigenesis and facilitate the progression to CRPC.⁶ Alterations in the *TP53* gene are also enriched in metastatic CRPC compared to primary tumors.⁷ Consistent with their relevance in advanced disease, the deletion of both *Pten* and *Trp53* in murine prostate using probasin (PB, alias *Pbsn*)-Cre4 led to the rapid development of adenocarcinoma and progression to fatal sarcomatoid carcinoma.⁸ Interestingly, the combined inactivation of *Trp53* and *Pten* in adult mouse prostate epithelium through inducible Nkx3.1^{CreERT2} resulted in accelerated progression to tumors resembling CRPC with neuroendocrine differentiation.⁹ While mutations in *KRAS* are infrequent, the activation of Ras signaling occurs frequently in advanced prostate cancer and has been associated with adverse outcomes.¹⁰ Experiments in mice have shown that Ras activation significantly accelerated *Pten*-loss-induced prostate tumor development, accompanied by epithelial–mesenchymal transition (EMT) and a high incidence of metastasis.¹¹ In addition, combined *Pten* deletion and Kirsten (K)-Ras activation in AR-ablated luminal stem/progenitor cells resulted in tumors with focal neuroendocrine differentiation.¹²

In this study, a mouse model of metastatic prostate cancer was generated through the deletion of *Trp53* combined with oncogenic *Kras*^{G12D} expression in the prostatic epithelium. These mice developed early-onset metastatic prostate cancer associated with down-regulation of AR expression and high-degree EMT. PI3K catalytic subunit γ (PIK3CG) was identified as a potential drug target for the treatment of AR-indifferent metastatic prostate cancer.

Materials and Methods

Mouse Strains

Mouse strains PB-Cre4, p53^{flox} (alias *Trp53*^{flox}) and *Kras*^{LSL-G12D} were received from NCI Mouse Repository (Frederick, MD) and The Jackson Laboratory (Bar Harbor, ME), and have been previously described.^{13–15} Compound mutant mice were maintained on a mixed FVB/C57BL/6 background. All procedures in mice were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center (Jackson, MS).

Histologic Examination and IHC Analysis

Histologic examination and immunohistochemistry (IHC) analysis were performed as per standard protocols. Prostatic tissues from at least three mice of each genotype were processed for hematoxylin and eosin staining, and representative images were acquired using an Eclipse 80i microscope (Nikon Instruments, Melville, NY). With approval from the institutional review board, a tissue microarray was constructed with 78 human prostatic tissues, in which 27 samples were from African American patients, and 53 samples were from Caucasian American patients. The primary antibodies used for immunostaining were cytokeratin 5 (K5; 1:200; catalog number PRB-160P; BioLegend, San Diego, CA), cytokeratin 8 (K8; 1:200; catalog number MMS-162P; BioLegend), aldehyde dehydrogenase (Aldh)-1 (1:100; catalog number ab52492; Abcam, Cambridge, MA), AR (1:200; catalog number sc-816; Santa Cruz Biotechnology, Dallas, TX), twist basic helix-loop-helix transcription factor (Twist; 1:100; catalog number sc-6070; Santa Cruz Biotechnology), vimentin (1:200; catalog number 5741; Cell Signaling Technology, Danvers, MA), c-Met (1:100; catalog number 19971-1-AP; Proteintech, Rosemont, IL), prostatic acid phosphatase (ACPP; 1:100; catalog number 15840-1-AP; Proteintech), and Pik3cg (1:100; catalog number 5405; Cell Signaling Technology).

Western Blot Analysis

Prostate tissues from two mice per genotype were homogenized and lysed in radioimmunoprecipitation assay buffer (Boston BioProducts, Ashland, MA) supplemented with protease inhibitor (Sigma-Aldrich, St. Louis, MO). Protein concentrations were measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA) and protein samples of an equivalent amount were loaded. Antibodies for probing specific proteins, as follows, were used: AR (1:1000; catalog number sc-816; Santa Cruz Biotechnology), E-cadherin (1:1000; catalog number 3195; Cell Signaling Technology), vimentin (1:1000; catalog number 5741; Cell Signaling Technology), Pik3cg (1:1000; catalog number 5405; Cell Signaling Technology), and β -actin (1:5000; catalog number sc-81178; Santa Cruz Biotechnology).

Cell Culture Experiment

Murine prostate cancer cell line #4700 was established from a primary prostate tumor harvested from the p53^{flox/flox}; *Kras*^{LSL-G12D/+}; PB-Cre4 (PKC) mouse. Briefly, tumors were dissociated in EpiCult-B medium containing collagenase/hyaluronidase (Stemcell Technologies, Vancouver, BC, Canada), and single-cell suspensions were prepared according to the manufacturer's protocols. Nonepithelial cells were removed using the EasySep mouse epithelial cell enrichment kit (Stemcell Technologies), and cancer cells were then cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal

Figure 1 A mouse model for metastatic prostate cancer through concurrent *Trp53* deletion and *Kras* activation. **A:** Kaplan-Meier survival curves of p53^{flx/flx};PB-Cre4 (PC), *Kras*^{LSL-G12D/+};PB-Cre4 (KC), and p53^{flx/flx};Kras^{LSL-G12D/+};PB-Cre4 (PKC) mice. **B–E:** Gross pathology of the prostate tumor (arrowhead in B) and metastatic growth on the diaphragm (arrowhead in C), pancreas (arrowhead in D), and intestine (arrowhead in E). **F–H:** Representative histologic examination of the anterior prostate glands from wild-type (WT; F), PC (G), and KC (H) mice at 3 months of age. **I–K:** Representative histologic examination of the primary prostate tumors from PKC mice at 2 to 3 months of age. The boxed area in I corresponds to the high-magnification images in J and K. **L–N:** Representative histologic examination of tumor metastases in PKC mice, including tumor cells invaded into the diaphragm (L), adhering to the pancreas (M), and the kidney (N). **O–Q:** Immunohistochemistry analysis for prostatic acid phosphatase (ACPP)-positive tumor cells (arrowheads) in the diaphragm (O), pancreas-associated tumor tissue (P), and kidney-associated tumor tissue (Q) in PKC mice. Scale bars: 50 μ m (F–N); 25 μ m (O–Q).

bovine serum. Culture media for human prostate cell lines were as follows: LNCaP, RPMI 1640; PC3, F12K; and DU145, DMEM, with 10% fetal bovine serum. Cells were treated with enzalutamide (catalog number S1250; Selleck

Chemicals, Houston, TX), AS-605240 (catalog number S1410; Selleck Chemicals), and MK-0752 (catalog number S2660; Selleck Chemicals), all dissolved in dimethyl sulfoxide. Controls were incubated with an equivalent amount of

Figure 2 Characterization of prostate tumors from $p53^{flox/flox}; Kras^{LSL-G12D/+}; PB-Cre4$ (PKC) mice. **A–C:** Immunofluorescence staining of cytokeratin 5 (K5; **A**), cytokeratin 8 (K8; **B**), and both (**C**), with DAPI nuclear counterstain, in prostate tumors from PKC mice. **Arrows** indicate strong K5 staining in the basal cells of ducts; **arrowheads**, weak K5 staining in a subset of tumor cells; **asterisk**, a small area showing weak K8 staining. **D–I:** Immunohistochemistry analysis of aldehyde dehydrogenase (Aldh1) (**D**), androgen receptor (AR) (**E** and **F**), twist basic helix-loop-helix transcription factor Twist (**G**), vimentin (**H**), and Met (**I**) in prostates from PKC mice. Immunostaining of AR is negative or very weak in the prostate tumor cells (**E**), whereas strong nuclear staining of AR is seen in the neighboring benign prostate tissue (**F**). **J:** Western blot analysis of AR, E-cadherin (E-Cad), and vimentin in prostates from wild-type (WT), $p53^{flox/flox}; PB-Cre4$ (PC), $Kras^{LSL-G12D/+}; PB-Cre4$ (KC), and PKC mice at 3 months of age. β -Actin is included as a loading control. **K:** Growth curves of prostate cancer cell lines LNCaP (left) and #4700 (derived from PKC mice; right) cells treated with enzalutamide (10 μ mol/L) or vehicle control (dimethyl sulfoxide). Relative numbers of viable cells at each time point were determined by MTS assay and are presented as means $\pm$ SD of the absorbance at 490 nm. **** $P < 0.0001$ versus control (Sidak multiple-comparisons test). Scale bars = 50 μ m (**A–I**). MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium inner salt.

vehicle alone. Cell growth was assessed using the Cell-Titer96 AQueous One solution kit (Promega, Madison, WI). For tumorsphere assay, tumor cells were plated in an ultralow attachment plate (Corning Life Sciences, Tewksbury, MA) at a concentration of 2000 cells/well, and expanded in serum-free DMEM/F12 medium containing mammary epithelial cell growth medium supplements and growth factors (catalog number CC-4136; Lonza, Basel, Switzerland) for 10 days. Spheres (diameter ≥ 50 μ m) were quantified using a Celigo cytometer (Nexcelom Bioscience, Lawrence, MA).

Statistical Analysis

Statistical analysis was performed using Prism software version 8.3.1 (GraphPad Software, San Diego, CA). The data are

expressed as means $\pm$ SD. Survival was calculated using the Kaplan-Meier method and compared by the log-rank test. Correlations analysis was performed using the χ^2 test. Two-group comparisons were analyzed using a two-tailed t -test as a post hoc for analysis of variance, with pertinent correction for multiple comparisons. $P \leq 0.05$ were considered statistically significant.

Results

Kras Activation Cooperates with p53 Deficiency to Induce Metastatic Prostate Cancer

A mouse model of metastatic prostate cancer was generated through PB-Cre4-directed deletion of *Trp53* and expression

Figure 3 Targeting *Pik3cg* in metastatic prostate cancer caused by *Trp53* deletion and *Kras* activation in mice. **A:** Representative photomicrograph (left) and quantitation (right) of *Pik3cg* immunohistochemistry analysis in the prostate tumors from PKC mice. **B:** Western blot analysis of *Pik3cg* in the prostates of wild-type (WT), *p53*^{flx/flx};PB-Cre4 (PC), *Kras*^{LSL-G12D/+};PB-Cre4 (KC), and *p53*^{flx/flx}; *Kras*^{LSL-G12D/+};PB-Cre4 (PKC) mice at 3 months of age. β -Actin is the loading control. **C:** Growth curves in PKC cell line #4700 treated with *Pik3cg* inhibitor AS-605240 or vehicle control (dimethyl sulfoxide; DMSO). **D:** Western blot analysis of E-cadherin and vimentin in #4700 cells with the treatment of AS-605240 or vehicle control. **E:** Representative photomicrographs (left) and quantitation (right) of tumorspheres formed in three-dimensional culture of #4700 cells in the presence of vehicle control (DMSO) or 20 μ mol/L AS-605240 (AS). **F:** Kaplan-Meier survival curves of PKC mice with or without the treatment of AS-605240. The treatment group received daily i.p. injection of AS-605240 (20 mg/kg body weight, dissolved in 0.5% carboxymethylcellulose and 0.25% Tween 20 saline at a final concentration of 4 mg/mL), 5 days a week for 5 weeks, starting from the age of 50 days. **G:** Quantitation of tumor metastasis in PKC mice with or without the treatment with AS-605240. Data are expressed as means $\pm$ SD (A, C, and E). $n = 3$ (A); $n = 3$ per group (C and E); $n = 8$ per group (F and G). *** $P < 0.001$, **** $P < 0.0001$ versus control at all time points (Tukey multiple-comparisons test); ††† $P < 0.001$ (unpaired *t*-test); † $P < 0.05$ (χ^2 test). Scale bars: 50 μ m (A); 100 μ m (E).

of the oncogenic *Kras*^{G12D} in the prostate epithelium. Highly aggressive metastatic prostate cancer developed in the PKC mice at 2 to 3 months of age, with a median survival of 80.5 days (Figure 1A). Metastatic prostate tumor was formed in some *p53*^{flx/+}; *Kras*^{LSL-G12D/+};PB-Cre4 mice after 6 months of age. For comparison, fatal tumors did not develop in either *p53*^{flx/flx};PB-Cre4 or *Kras*^{LSL-G12D/+};PB-Cre4 mice (Figure 1A). In the vast majority of PKC mice, ascites developed, as well as metastasis to a variety of tissues/organs including the diaphragm, pancreas, intestine, and kidney (Figure 1, B–E). In agreement with the findings from a previous study in which the deletion of *Trp53* alone in the prostate was not associated with pathologic changes,¹⁶ prostates in *p53*^{flx/flx};PB-Cre4 mice showed the same morphology as did those in wild-type mice at 3 months of age (Figure 1, F and G). Although no lesions were reported in mice with prostatic expression of *Kras*^{G12D} through either PB-Cre4 or Nkx3.1-Cre,^{11,17} a small number of prostatic intraepithelial neoplasia lesions were noted in *Kras*^{LSL-G12D/+};PB-Cre4 mice at this age (Figure 1H). The prostate tumors in PKC mice mainly consisted of spindle-shaped/mesenchymal-like cells (Figure 1,

I–K), resembling the sarcomatoid carcinoma resulting from synchronous deletion of the *Pten* and *Trp53* tumor suppressor genes.⁸ The metastatic growth in the diaphragm was manifested by the intermingling of tumor cells with the muscle cells (Figure 1L). However, tumors spreading to the pancreas and kidney were largely superficial, as there remained a boundary between tumor cells and destination tissues (Figure 1, M and N). A subset of tumor cells in the diaphragm, pancreas, and kidney stained positive for a prostate-specific marker, ACPP, supporting their prostatic origin (Figure 1, O–Q). Taken together, oncogenic *Kras* activation and *p53* deficiency cooperate to induce early-onset metastatic prostate cancer in mice.

PKC Tumors Are AR-Negative Adenocarcinoma with Extensive EMT

PKC tumors were characterized by immunostaining for various markers of the prostatic epithelium. While the basal layer in the glandular structures showed intense staining of

Figure 4 Expression of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma (PIK3CG) in human prostate cancers. **A–D:** Representative photomicrographs of PIK3CG immunostaining on a human prostatic tissue microarray, including specimens of benign/hyperplastic prostatic tissue (**A**) and low-grade (**B**), high-grade (**C**), and metastatic (**D**) prostate cancer. **Inset:** High magnification of staining. **Arrowheads** indicate tumor cells with positive PIK3CG staining. **E:** Quantitation of PIK3CG immunostaining in four pathologic groups. **F:** Western blot analysis of PIK3CG (top) and androgen receptor (AR) (bottom) in PC3, DU145, and LNCaP cells treated with MK-0752 (top) or AS-605240 (bottom) (both, 20 μ mol/L) or equal volume of vehicle control (dimethyl sulfoxide). **G:** Growth curves of PC3 (left), DU145 (center), and LNCaP (right) cells treated with AS-605240 (20 μ mol/L) or control. Relative numbers of viable cells were determined by MTS assay and are presented as means $\pm$ SD of the absorbance at 490 nm. $n = 11$ (**B**); $n = 14$ (**D**); $n = 20$ (**A**); $n = 25$ (**C**). * $P < 0.05$ among all four groups (χ^2 test); $^{\dagger}P < 0.05$; $^{\ddagger}P < 0.01$, $^{\text{†††}}P < 0.001$, and $^{\text{††††}}P < 0.0001$ versus control (Sidak multiple-comparisons test). Scale bars = 50 μ m. PIK3CG (+), $\geq 1\%$ positive cells; PIK3CG (–), 0% positive cells. B, benign; H, high-grade; L, low-grade; M, metastatic; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt.

the basal cell marker K5, a subset of tumor cells showed weak K5 staining. Weak staining of the luminal cell marker K8 was also noted in a subset of tumor cells; however, there was no overlap of K5 and K8 staining (Figure 2, A–C). Stem and progenitor-like cells of the prostate have shown high-level Aldh activity in mice.¹⁸ The PKC tumors contained ductal cells that highly expressed Aldh1, but the rest of the poorly differentiated tumor cells were Aldh1 negative (Figure 2D). AR-mediated signaling has been shown to play a central role in the initiation and progression of prostate cancer. Intriguingly, PKC tumors stained negative for AR (Figure 2E), whereas the neighboring benign tissue remained AR positive (Figure 2F). Since the majority of PKC tumor cells are spindle-shaped/mesenchymal-like, they might have undergone EMT, a process associated with increased tumor

aggressiveness.¹⁹ Indeed, they stained positive for the mesenchymal marker vimentin as well as Twist, a key transcription factor for EMT (Figure 2, G and H). In addition, PKC tumors showed high-level expression of c-Met, a receptor tyrosine kinase that has been associated with hormone-refractory and metastatic prostate cancer and is amplified in prostate tumors in $p53^{\text{flox/flox}}$, $Pten^{\text{flox/flox}}$, PB-Cre4 mice^{20,21} (Figure 2I). Western blot analysis confirmed the decreased expression of AR and up-regulation of vimentin in PKC prostates. Interestingly, $Kras^{\text{LSL-G12D/+}}$, PB-Cre4 prostates showed elevated E-cadherin, whereas PKC prostates almost lost E-cadherin (Figure 2J). Of note, the deletion of *Cdh1* (encoding E-cadherin) in prostatic epithelial cells was recently shown to induce prostatic intraepithelial neoplasia formation, and the combined deletion of *Cdh1* and *Pten*

resulted in early-onset aggressive prostate cancer in mice.²² Collectively, p53 loss combined with *Kras*^{G12D} in the murine prostate resulted in metastatic prostate cancer with characteristics of EMT and down-regulation of AR. Enzalutamide is a new anti-androgen agent that has been shown to prolong survival among patients with metastatic CRPC. Therefore, the sensitivity of PKC tumor cells to enzalutamide was tested using a prostate cancer cell line (#4700) established from the PKC mouse. While the growth of the androgen-sensitive prostate cancer cell line LNCaP was inhibited by enzalutamide, PKC cell line #4700 was insensitive to enzalutamide (Figure 2K). These findings suggest that PKC tumors are resistant to AR inhibition; the identification of a new therapeutic target is needed for this type of prostate cancer.

Targeting PIK3CG in AR-Indifferent Metastatic Prostate Cancer

PKC tumors display a mesenchymal feature similar to that of claudin-low tumors of the breast and bladder.^{23,24} *PIK3CG*, encoding the sole class IB PIK3, is upregulated specifically in the claudin-low subtype of human breast cancer, where it promotes tumor cell proliferation, migration, and invasion.²⁵ Noteworthy, whole-exome sequencing detected *PIK3CG* amplification in metastatic biopsy samples from up to 24% of CRPC patients.²⁶ Advanced prostate cancer with mismatch repair defects also showed overexpression of PIK3CG.²⁷ In addition, a clonal truncal mutation in *PIK3CG* was found at all metastatic sites (but not at the primary site) in a prostate cancer patient, suggesting a specific role for this gene in metastatic disease.²⁸ Given these findings, Pik3cg expression and its potential role were explored in PKC mice. As expected, IHC analysis showed that about 10% of PKC tumor cells stained positive for Pik3cg (Figure 3A). Pik3cg was undetectable by Western blot analysis in the prostates of wild-type, p53^{flox/flox};PB-Cre4, and *Kras*^{LSL-G12D/+};PB-Cre4 mice, but were readily detected in PKC prostates (Figure 3B). Next, the function of Pik3cg in PKC tumor cells was tested using the Pik3cg inhibitor AS-605240, which has been shown to selectively inhibit Pik3cg, over 30- and 7.5-fold more selective for Pik3cg than Pi3k δ/β and Pi3k α , respectively, in cell-free assays.²⁹ With treatment of the PKC cell line with AS-605240, cell growth was almost completely suppressed (Figure 3C). Moreover, with the inhibition of Pik3cg, EMT was reversed, as evidenced by increased E-cadherin and decreased vimentin protein levels (Figure 3D). Tumorsphere assay was also performed using #4700 cells incubated with AS-605240 or vehicle as control. Treatment with AS-605240 was associated with a drastically decreased capacity of tumorsphere formation (Figure 3E), which could conceivably reflect a reduction in the number of cancer stem/tumor-initiating cells. These findings suggest that pharmacologic inhibition of Pik3cg not only reversed the EMT phenotype of PKC tumor cells, but also inhibited the proliferation of these cells, including cancer stem cells. Next, the

therapeutic efficacy of AS-605240 was tested by the treatment of PKC mice for up to 5 weeks, starting from 7 weeks of age. The treatment was associated with a statistically nonsignificant increase in median survival (from 80.5 to 93.5 days) (Figure 3F), which was not as robust as *in vitro* proliferation and tumorsphere assays. Interestingly, a significantly lower percentage of animals in the AS-605240-treated group showed metastasis compared with that in the untreated group (Figure 3G), suggesting that Pik3cg may be more important in prostate cancer metastasis than in tumor cell growth and survival. Taken together, the present findings suggest that PIK3CG plays an important role in prostate cancer associated with p53 loss and KRas activation, and that targeting PIK3CG may represent an effective therapeutic strategy for advanced metastatic diseases.

Expression of PIK3CG in Human Prostate Cancer Cells

For an understanding of the putative role of PIK3CG in human prostate cancer, IHC analysis of PIK3CG was performed in a tissue microarray that included benign prostate tissue, low- and high-grade prostate adenocarcinomas, and metastatic tumors from patients. Comparison of the staining among the four pathologic groups showed dynamic expression of PIK3CG during the initiation, progression, and metastasis of human prostate cancer. All 20 of the benign/hyperplastic specimens stained completely negative for PIK3CG (Figure 4A), and 1 of 11 low-grade adenocarcinomas showed positive staining in a subset of tumor cells (Figure 4B). Interestingly, 9 of 25 high-grade and 4 of 14 metastatic specimens showed robust PIK3CG staining (Figure 4, C and D). A χ^2 test found that the positivity for PIK3CG in prostate cancer specimens was significantly associated with advanced clinical stages (Figure 4E), suggesting that PIK3CG may be involved in the progression and metastasis of human prostate cancer. Next, PIK3CG expression was examined in human prostate cancer cell lines. Western blot analysis readily detected a band of PIK3CG in PC3 and LNCaP cells (with a higher level in the latter), but barely in DU145 cells (Figure 4F). Consistent with the PIK3CG expression levels in the three cell lines, AS-605240 showed an inhibitory effect on the growth of these cells, more significantly in LNCaP and PC3 (Figure 4G). Although PIK3CG expression has been shown to be regulated by neurogenic locus notch homologue protein (Notch) in claudin-low breast cancer cells,²⁵ the inhibition of Notch signaling using MK-0752 in prostate cancer cells had no effect on PIK3CG protein levels (Figure 4F), suggesting tissue specificity of Notch regulation on PIK3CG. Decreased AR protein level has been noted in human and murine prostate tumors with *PTEN* deletion, with inhibition of the PI3K pathway resulting in increased AR protein levels.³⁰ Western blot analysis of AR showed high-level expression in LNCaP but undetectable levels in PC3 and DU145, and that inhibiting PIK3CG had no effect on AR protein level (Figure 4F). To summarize, PIK3CG was expressed in

human prostate cancers, especially in high-grade and metastatic disease, and pharmacologic inhibition of PIK3CG had an inhibitory effect on cancer cell growth.

Discussion

It is clear that pharmacologic inhibition of PIK3CG blocks tumor cell growth and reverses EMT *in vitro*. Despite a substantial decrease in metastasis, PKC mice treated with the Pik3cg inhibitor had only a slight gain in median survival. Postmortem examination found severely dilated bladder filled with urine in almost all PKC mice (data not shown), suggesting urinary obstruction caused by extensive local tumor invasion. Bladder/ureteral obstruction can lead to hydronephrosis and kidney failure, which may be the cause of death in these mice. Of note, spontaneous death from urinary obstruction has been reported in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model.^{31,32} It appears that the inhibition of Pik3cg suppresses metastasis, with a minimal effect on primary tumor growth (and urinary obstruction), suggesting a role of Pik3cg that is more important in the spread than in the growth of prostate cancer cells.

To our knowledge, there has been no report of p53 loss and KRAS activation in human prostate cancer leading to AR-negative tumor and EMT phenotype. Interestingly, the prostate cancer cell lines DU145 and PC3, originally derived from prostate cancer metastasis to the brain and bone, respectively, express high-level KRAS and either mutant or no p53, and are both AR negative.^{33,34} An oncogenic gene fusion of KRAS has also been identified in the DU145 cell line.³⁵ The EMT-like phenotype and metastatic behavior of prostate cancer induced by concurrent Kras activation and p53 depletion in mice were recently described by Weng et al.³⁶ The cancer stemness gene CD24 was identified as a key driver of tumorigenesis and metastasis in their study.³⁶ In the present article, p53 loss and oncogenic Kras were shown to cooperatively induce AR-negative metastatic prostate cancer, associated with up-regulation of Pik3cg. Decreased AR expression/signaling in prostate cancer has been documented in several mouse models. While AR expression was highly heterogeneous in prostate tumors from *Pten*^{flox/+}; *Kras*^{LSL-G12D/+}; PB-Cre mice, prostates in *Pten*^{flox/flox}; *Kras*^{LSL-G12D/+}; PB-Cre mice showed reduced expression of AR target genes compared to that in *Pten*^{flox/flox}; PB-Cre prostates.¹¹ Triple deletion of *Pten*, *Rb1*, and *Trp53* in the mouse prostate has been reported to result in castration-resistant prostate tumors, with decreased levels of AR expression but elevated levels of a neuroendocrine lineage marker.³⁷ The emergence of an AR-null and neuroendocrine-null phenotype has also been reported in prostate cancer patients following androgen-deprivation therapy.³⁸ These tumors display elevated fibroblast growth factor (FGF) and mitogen-activated protein kinase (MAPK) pathway activity, and targeting these two pathways has been suggested for prostate cancers resistant to AR-targeted therapy.³⁸ The present study discovered an oncogenic role of Pik3cg in AR-negative

prostate cancer, where it functions within the tumor cells to promote survival and EMT. Interestingly, an earlier study found that PIK3CG activation in myeloid cells promoted tumor inflammation, while its inhibition blocked tumor inflammation, growth, and progression.³⁹ In addition, Pik3cg has been shown to drive immunosuppressive programming in tumor-associated macrophages, a phenomenon that inhibits adaptive immune responses.⁴⁰ Conversely, inhibiting Pik3cg was associated with reprogramming of tumor-associated macrophages to stimulate T-cell-mediated tumor suppression in mouse models of several cancers.^{40,41} Thus, targeting PIK3CG may improve the treatment of advanced prostate cancer through actions on both tumor cells and tumor microenvironment.

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