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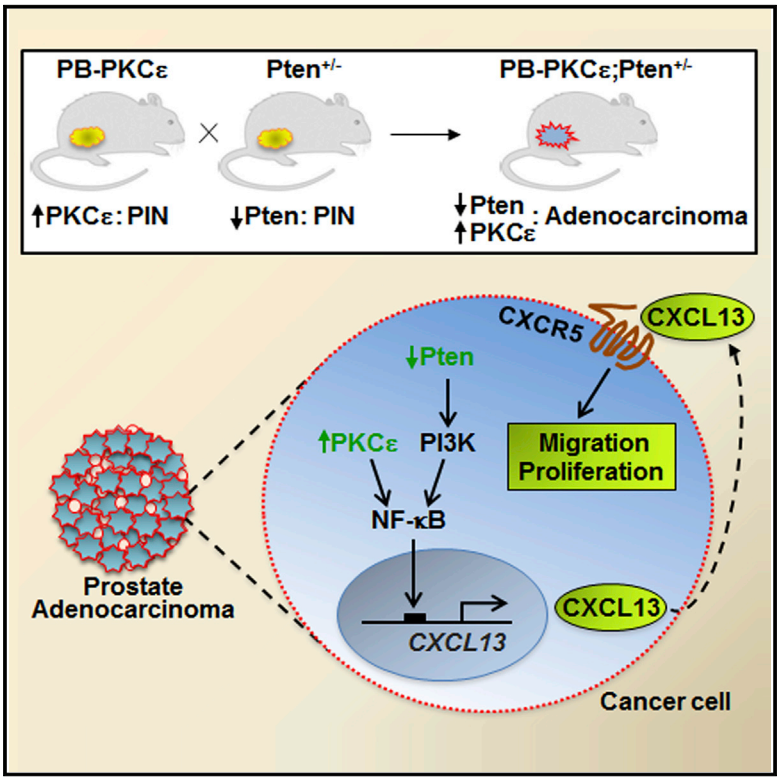
Protein Kinase C Epsilon Cooperates with PTEN Loss for Prostate Tumorigenesis through the CXCL13-CXCR5 Pathway

Graphical Abstract



Authors

Rachana Garg, Jorge M. Blando, Carlos J. Perez, Martin C. Abba, Fernando Benavides, Marcelo G. Kazanietz

Correspondence

marcelog@upenn.edu

In Brief

Garg et al. find that PKCε overexpression cooperates with Pten loss to promote prostate cancer in mice. These two alterations together confer enhanced growth, tumorigenic, migratory and invasive capabilities to prostate epithelial cells, and promote the release of CXCL13, an effect that is mediated by the non-canonical NF-κB pathway.

Highlights

- PKCε overexpression in a Pten-deficient background promotes prostate cancer in mice
- This cooperation confers a highly proliferative, migratory, and invasive phenotype
- PKCε overexpression with Pten loss leads to CXCL13 upregulation via NF-κB
- Elevated CXCL13 mediates migratory and tumorigenic activity of prostate cancer cells

Accession Numbers

GSE86257

Garg et al., 2017, Cell Reports *19*, 375–388
April 11, 2017
http://dx.doi.org/10.1016/j.celrep.2017.03.042

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Protein Kinase C Epsilon Cooperates with PTEN Loss for Prostate Tumorigenesis through the CXCL13-CXCR5 Pathway

Rachana Garg,[1] Jorge M. Blando,[2] Carlos J. Perez,[3,4] Martin C. Abba,[5] Fernando Benavides,[3,4] and Marcelo G. Kazanietz[1,6,*]

[1]Department of Systems Pharmacology and Translational Therapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA
[2]Immunopathology Laboratory, Department of Immunology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA
[3]Department of Epigenetics and Molecular Carcinogenesis, The University of Texas MD Anderson Cancer Center, Smithville, TX 78957, USA
[4]The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, TX 77030, USA
[5]Centro de Investigaciones Inmunológicas Básicas y Aplicadas-CONICET, Universidad Nacional de La Plata, CP1900 La Plata, Argentina
[6]Lead Contact
*Correspondence: marcelog@upenn.edu
http://dx.doi.org/10.1016/j.celrep.2017.03.042

SUMMARY

PKCε, an oncogenic member of the PKC family, is aberrantly overexpressed in epithelial cancers. To date, little is known about functional interactions of PKCε with other genetic alterations, as well as the effectors contributing to its tumorigenic and metastatic phenotype. Here, we demonstrate that PKCε cooperates with the loss of the tumor suppressor *Pten* for the development of prostate cancer in a mouse model. Mechanistic analysis revealed that PKCε overexpression and Pten loss individually and synergistically upregulate the production of the chemokine CXCL13, which involves the transcriptional activation of the CXCL13 gene via the non-canonical nuclear factor κB (NF-κB) pathway. Notably, targeted disruption of CXCL13 or its receptor, CXCR5, in prostate cancer cells impaired their migratory and tumorigenic properties. In addition to providing evidence for an autonomous vicious cycle driven by PKCε, our studies identified a compelling rationale for targeting the CXCL13-CXCR5 axis for prostate cancer treatment.

INTRODUCTION

Since its identification as the main intracellular receptor for the phorbol ester tumor promoters, protein kinase C (PKC) has been widely implicated in cancer progression. The PKC family of Ser/Thr kinases has been categorized into classical (cPKCs α, βI, βII, and γ), novel (nPKCs δ, ε, η, and θ), and atypical (aPKCs ζ and λ/ι) groups. cPKCs and nPKCs, the phorbol-ester-regulated PKCs, are physiologically activated by diacylglycerol (DAG), a lipid second messenger generated by activation of extracellular receptors (Griner and Kazanietz, 2007; Rosse et al., 2010). The diverse functional specificity of individual PKC isozymes (i.e., tumor promoters versus tumor suppressors) reflects their cell-type-specific idiosyncratic regulation of oncogenic and growth-inhibitory signaling pathways. Altered patterns of isozyme expression and/or activation status are often linked to promotion or suppression of the cancer phenotype (Garg et al., 2014; Murray et al., 2011).

Among the multiple PKCs, PKCε emerged as a pro-oncogenic kinase and tumor biomarker. PKCε upregulation has been reported in a number of cancer types, potentially reflecting its involvement in disease etiology and progression (Aziz et al., 2007; Griner and Kazanietz, 2007; Jain and Basu, 2014; Pan et al., 2005). Growth-promoting, survival, and transforming roles for PKCε have been identified in numerous cellular models. Consistent with these effects, PKCε activates mitogenic and survival pathways, namely Ras/Erk, phosphatidylinositol 3-kinase (PI3K)/Akt, nuclear factor κB (NF-κB), and Stat3 (Aziz et al., 2007; Benavides et al., 2011; Garg et al., 2014; Jain and Basu, 2014; McJilton et al., 2003; Meshki et al., 2010; Mischak et al., 1993). PKCε also emerged as a positive regulator of cancer cell motility, invasion, and epithelial-mesenchymal transition (EMT) (Caino et al., 2012b; Garg et al., 2014; Jain and Basu, 2014). Accordingly, pharmacological inhibition or RNAi silencing of PKCε impairs cancer cell growth in culture and as xenografts and prevents their metastatic dissemination (Aziz et al., 2007; Caino et al., 2012a; Pan et al., 2005). Notwithstanding, the molecular mechanisms and downstream effectors behind the tumorigenic and metastatic activities of PKCε remain only partially understood.

Emerging evidence links PKCε to prostate cancer progression. PKCε is essentially undetectable in normal or benign prostate epithelium; however, it is highly expressed in most human prostate tumors and recurrent disease (Aziz et al., 2007; Cornford et al., 1999; McJilton et al., 2003). Spontaneous prostate tumors formed in TRAMP mice, and their metastases are impaired upon genetic ablation of the PKCε gene (*Prkce*) (Hafeez et al., 2011). Notably, transgenic overexpression of PKCε in the mouse prostate leads to preneoplastic lesions; however, it is insufficient to confer a complete cancer phenotype (Benavides et al., 2011).

The nature of PKCε effectors responsible for the acquired prostate phenotype and the functional interaction with relevant oncogenic/tumor suppressing inputs remain poorly understood.

PI3K is another pathway widely implicated in the progression of prostate cancer. *PIK3CA* gene amplification and mutations can be detected in advanced prostate tumors (Agell et al., 2011; Robinson et al., 2015; Sarker et al., 2009; Sun et al., 2009). However, the most common alteration in this pathway is the loss of PTEN, a phosphatase for the PI3K product PIP3. PTEN gene deletions and inactivating mutations are commonly observed in prostate tumors and their metastases (Sarker et al., 2009). Not surprisingly, loss of a single *Pten* allele confers preneoplastic lesions, whereas conditional deletion of both *Pten* alleles leads to metastatic prostate cancer (Blando et al., 2011; Di Cristofano et al., 1998; Kim et al., 2002; Podsypanina et al., 1999; Zhong et al., 2006). Here, we report that PKCε overexpression and Pten loss functionally interact for the development of prostate cancer in a mouse model, and we identified C-X-C motif chemokine 13 (CXCL13) as a bona fide effector of PKCε in prostate cancer, thus establishing a molecular paradigm in the progression of this disease.

RESULTS

PKCε Overexpression Cooperates with Pten Loss to Promote Prostate Cancer

Prostate-specific overexpression of PKCε in mice under the control of rat probasin (PB) promoter (PB-PKCε) confers prostatic intraepithelial neoplasia (PIN) lesions that do not progress to malignancy (Benavides et al., 2011). As *PTEN* loss of function is a frequent event in human prostate cancer, we intercrossed our transgenic PB-PKCε mice with mice heterozygous for *Pten* ($Pten^{+/-}$), which also display prostate preneoplastic lesions (Blando et al., 2011; Di Cristofano et al., 1998; Zhong et al., 2006). Remarkably, in addition to hyperplasia and PIN lesions, the resulting compound mutant mice (PB-PKCε;$Pten^{+/-}$) developed well-differentiated prostatic adenocarcinomas (ACs), preferentially in the ventral prostate, with an incidence of ~64% at 12 months (Figures 1A and 1B). No other lesions could be detected in the remaining of the genito-urinary track. ACs in PB-PKCε;$Pten^{+/-}$ mice display tubule and acinar structures and show in some cases evident stromal invasion (Figure S1A). PIN lesions in the compound mice presented cribriform patterns; cells display karyomegaly and cytomegaly and an enlarged nucleus with apical localization, and two or more macro-nucleoli were also observed. ACs showed the presence of macro-nucleoli, nucleus with enlarged shapes, and evident neovascularization. Thus, PKCε overexpression and Pten loss cooperate for the development of prostate cancer in mice. In line with these findings, Kaplan-Meier and Cox univariate regression analyses using the Cancer Genome Atlas (TCGA) RNA-sequencing (RNA-seq) dataset obtained from the Cancer Genomics Browser (https://genome-cancer.soe.ucsc.edu) show the worst prognosis for prostate cancer patients with high PKCε and low PTEN levels (Figure S1B).

As previously observed (Benavides et al., 2011), immunohistochemical analysis of PINs from PB-PKCε mice revealed activation of Akt and its effectors mTOR and S6. A similar effect was seen in PINs from $Pten^{+/-}$ mice. Prostatic ACs in PB-PKCε;$Pten^{+/-}$ compound mice display stronger staining for these markers (Figure 1C). Consistent with the role of PKCε in Stat3 activation in prostate cancer (Aziz et al., 2007; Hafeez et al., 2011), significant phospho-Stat3 staining was observed in PIN lesions and ACs, particularly in the nucleus.

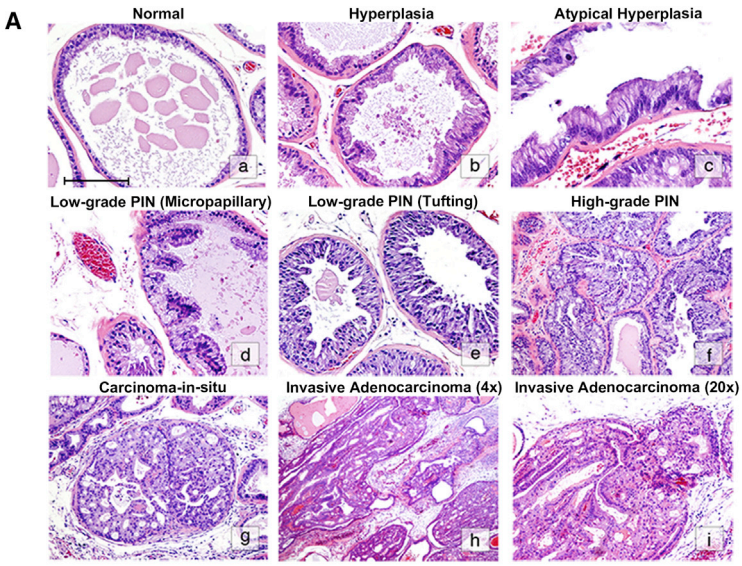
Cooperation of PKCε Overexpression and Pten Loss for Growth and Tumorigenesis

To assess the mechanisms underlying the observed cooperativity between PKCε overexpression and Pten loss in prostate cancer, we took advantage of isogenic murine prostate epithelial cell lines derived from Pten knockout (KO) mice that either express (P2 and P8) or do not express Pten (CaP2 and CaP8) (Jiao et al., 2007). Cells were stably transduced with lentiviruses for either PKCε or LacZ (LZ; control) (Figure 2A). P2 and P8 cells overexpressing PKCε (P2-PKCε and P8-PKCε) proliferate at higher rates than the corresponding control cell lines (P2-LZ and P8-LZ), while CaP2 and CaP8 cells displayed slightly elevated proliferation rates. A large proliferative advantage was observed in Pten null cells subject to PKCε overexpression (CaP2-PKCε and CaP8-PKCε), which became particularly evident in clonogenic and anchorage-independent growth assays (Figures 2B, 2C, and S2A). Remarkably, only CaP8-PKCε cells display tumorigenic activity upon subcutaneous (*s.c.*) inoculation into nude mice (Figure 2D). A bulbous hyperplastic overgrowth was observed for CaP8 and P8-PKCε cells; however, these cell lines did not form tumors in nude mice.

Next, we assessed the effect of PKCε overexpression on relevant proliferation and survival signaling pathways. Consistent with our previous findings in "normal" immortalized human RWPE1 prostate epithelial cells (Benavides et al., 2011), PKCε overexpression in P8 cells led to a small increase in basal and epidermal growth factor (EGF)-stimulated phospho-Erk, phospho-Akt, and phospho-mTOR levels. Most notably, CaP8-PKCε cells displayed higher basal as well as EGF-stimulated activation of Erk, Akt, and mTOR (Figure 2E). Platelet-derived growth factor (PDGF) and insulin growth factor 1 (IGF-1) stimulation led to comparable effects (Figure S2B). Taken together, these results indicate a strong cooperativity between PKCε overexpression and Pten loss for growth/survival signaling and tumorigenesis.

PKCε Overexpression Promotes Migratory and Invasive Phenotypes

Emerging evidence linked PKCε with cancer cell migration and invasiveness in vitro and in vivo (Garg et al., 2014). We found that migration of P8-PKCε and CaP8 cells in response to fetal bovine serum (FBS), IGF-1, or PDGF was significantly higher than parental P8 cells, as assessed with a Boyden chamber. Interestingly, a remarkably high migratory response to stimuli was observed in CaP8-PKCε cells (Figure 2F), which was also evident in wound assays (Figure S3). Assessment of the invasive properties revealed similar results, with CaP8-PKCε cells displaying the strongest invasive potential (Figure 2G). Hence, overexpression of PKCε in a Pten null background caused a dramatic enhancement in migratory and invading capabilities of prostate epithelial cells.



B

Line	Prostate	Hyperplasia	PIN	Adenocarcinoma
PB-PKCε	Ventral	12/12 (100%)	8/12 (66%)	0/12 (0%)
	Dorsal	7/12 (58%)	3/12 (25%)	0/12 (0%)
Pten+/-	Ventral	4/4 (100%)	2/4 (50%)	0/4 (0%)
	Dorsal	4/4 (100%)	2/4 (50%)	0/4 (0%)
PB-PKCε;Pten+/-	Ventral	15/17 (88%)	15/17 (88%)	11/17 (64%)
	Dorsal	13/17 (76%)	7/17 (41%)	4/17 (23%)

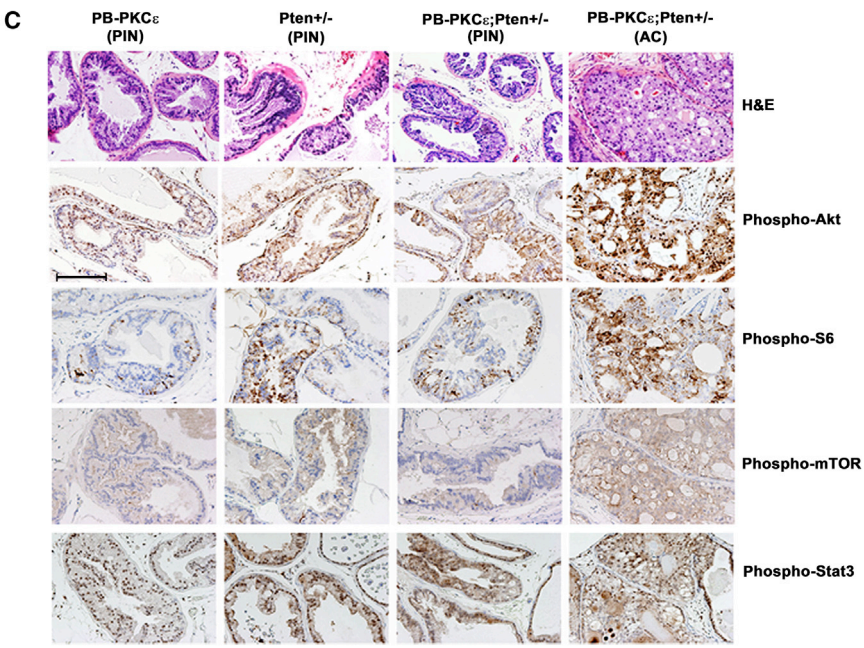


Figure 1. Phenotypes of Male PB-PKCε and PB-PKCε;Pten$^{+/-}$ Mice

(A) H&E staining in ventral prostates from 12-month-old normal wild-type mice (a) and PB-PKCε;Pten$^{+/-}$ mice (b–i). Magnification 20×, unless otherwise indicated. Scale bar, 100 μm.

(B) Incidence of lesions in 12-month-old mice.

(C) Immunohistochemical analysis of signaling markers. Scale bar, 100 μm.

Transcriptome Analysis of PKCε-Overexpressing Cells

To search for the molecular mechanisms underlying the observed phenotypes, a global gene expression profiling was carried out. Using the rank product test (q-value < 0.05), we identified 898, 573, and 1,101 differentially expressed genes associated with PKCε overexpression, Pten loss, and both alterations together, respectively, with some degree of overlap (Figure 3A; full list in Table S1). This analysis also revealed 187 genes (86 upregulated and 101 downregulated) that were commonly altered in CaP8, P8-PKCε, and CaP8-PKCε cells (Figures S4A–S4C). The top upregulated candidate among the differentially expressed genes categorized in CaP8-PKCε versus P8 group was *CXCL13* (fold change = 5.5, q < 0.0001), the gene coding for C-X-C motif chemokine 13. *CXCL13* was also among the top upregulated genes in P8-PKCε (fold change = 3.8, q < 0.0001) and CaP8 cells (fold change = 2.9, q < 0.0001) (Figure 3B). Other prostate-cancer-related genes that were commonly deregulated in the three genetic backgrounds include *ITGB8*, *LGR4*, *GPNMB*, and *MMP2.*

Analysis using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases uncovered major changes in pathways involved in cell proliferation, migration, adhesion, angiogenesis, and metabolism (Figure 3C). Comparison of biological processes enriched among the three cell lines depicts not only significant overlapping but also unique pathways ensuing PKCε overexpression and Pten loss (Figure 3D). An additional analysis of the top 50 deregulated transcripts (FC > 2, *q*-values < 0.000001) using Cytoscape and CluePedia identified a network of functionally enriched KEGG pathways specifically pertaining to PI3K-Akt signaling, GPCR-ligand binding, extracellular matrix organization, and cytokine-cytokine receptor interaction (Figure 3E). A comparative analysis of CaP8-PKCε-associated genes with the GSE6919 database identified 189 genes commonly deregulated between

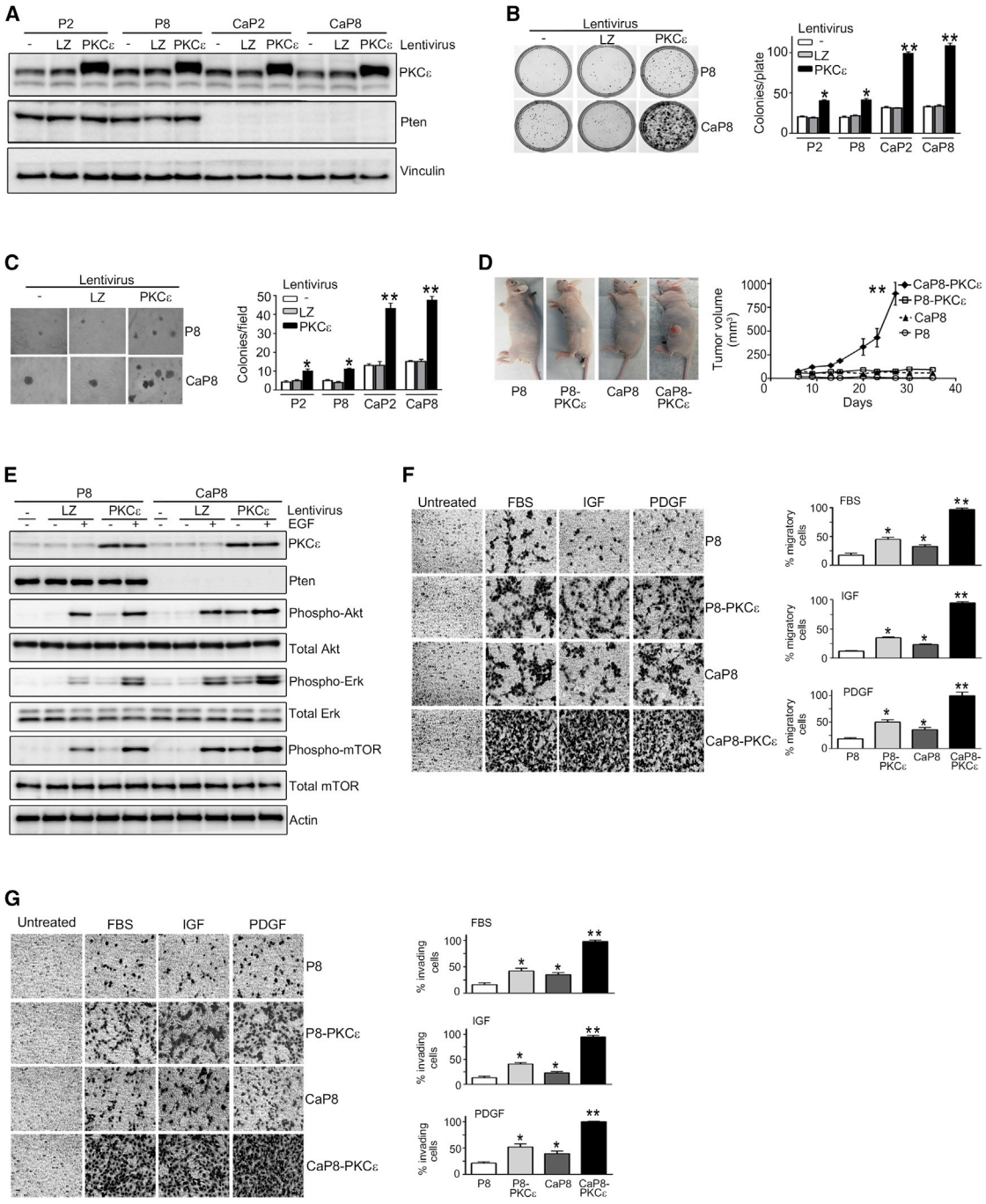


Figure 2. PKCε Overexpression and Pten Deletion Cooperate for Growth, Motility, and Invasion

(A) PKCε overexpression in P2, P8, CaP2, and CaP8 cells was achieved with a PKCε lentivirus. Control cells were infected with a LacZ (*LZ*) lentivirus.

(B) Clonogenic assay. Colony formation was assessed 15 days after cell seeding. Left: representative experiment. Right: quantification of colonies per plate.

(C) Anchorage-independent growth in soft agar was determined 21 days after seeding. Left: representative experiment. Right: quantification of colonies per field (five independent fields were counted and averaged).

(D) Tumor formation in athymic nude mice. Left: representative pictures 35 days after s.c. inoculation. Right: Tumor volume, expressed as mean ± SD (n = 5 mice/group).

(E) Activation of Akt, Erk, and mTOR in response to EGF (3 ng/mL, 2 min) as determined by western blot.

(F) Migration in the Boyden chamber in response to FBS (5%), IGF (50 ng/mL), or PDGF (50 ng/mL) was determined 16 hr after seeding. Left: representative images. Right: quantification of migrating cells by contrast microscopy in five independent fields. Results are expressed as mean ± SD of triplicate measurements. Two experiments gave similar results.

(G) Invasion was determined as in (F) but with Matrigel.

In (B)–(G), *p < 0.05 and **p < 0.01.

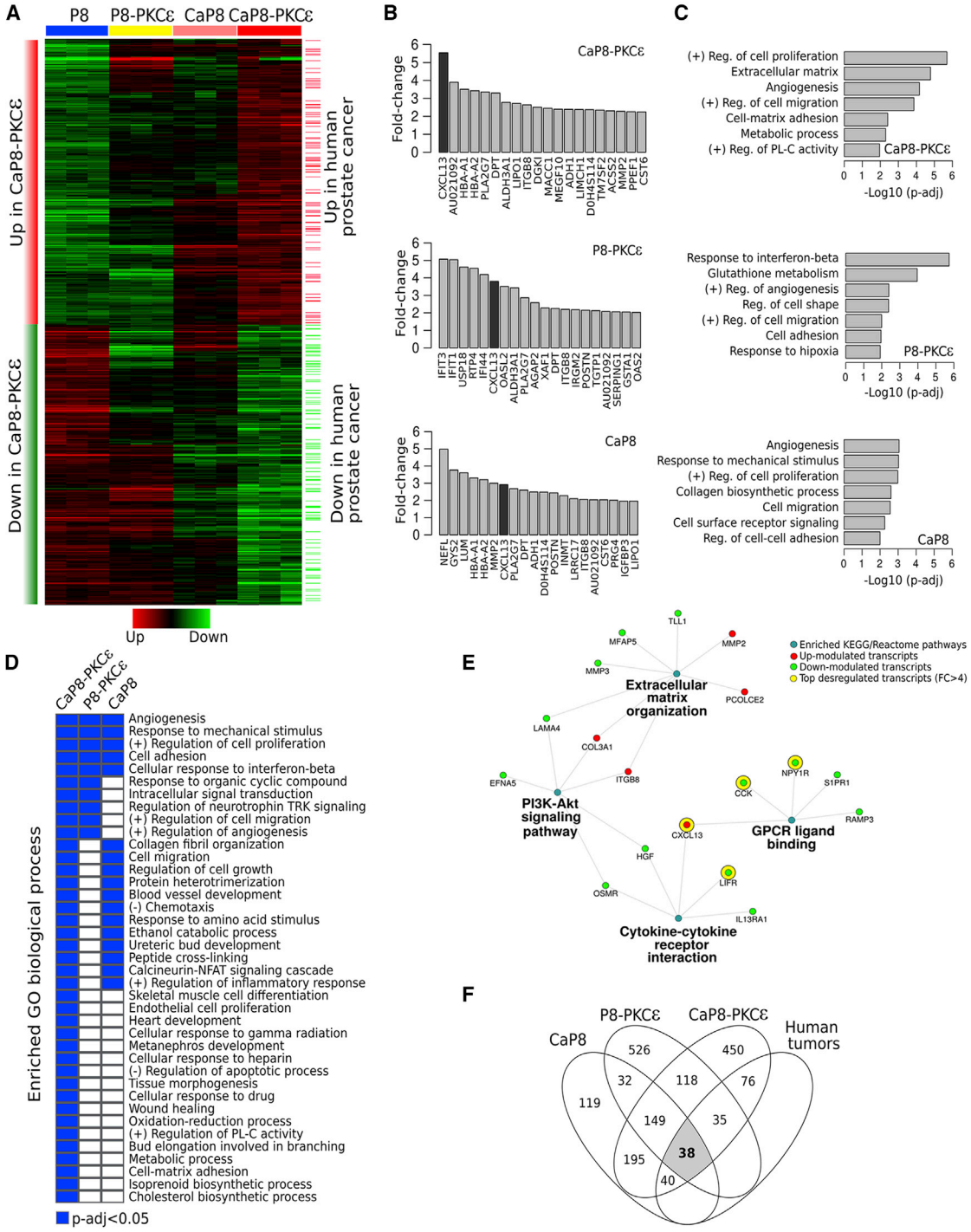


Figure 3. Gene Expression Profiling of PKCε-Overexpressing Prostate Epithelial Cells

(A) Heatmap of deregulated genes in murine cellular models. Red and green lines on the right indicate genes up- or down-modulated, respectively, in human primary and metastatic prostate tumors.

(B) Top 20 up-modulated genes among murine cell lines.

(C) Functional enrichment analysis of differentially expressed genes across the different cell models.

(D) Comparative analysis of enriched biological pathways/functions.

(E) CluePedia network of functionally enriched pathways and genes differentially expressed in CaP8-PKCε versus P8 cells.

(F) Euler diagram of transcripts commonly expressed among murine cell lines and human prostate cancer (GSE6919 dataset).

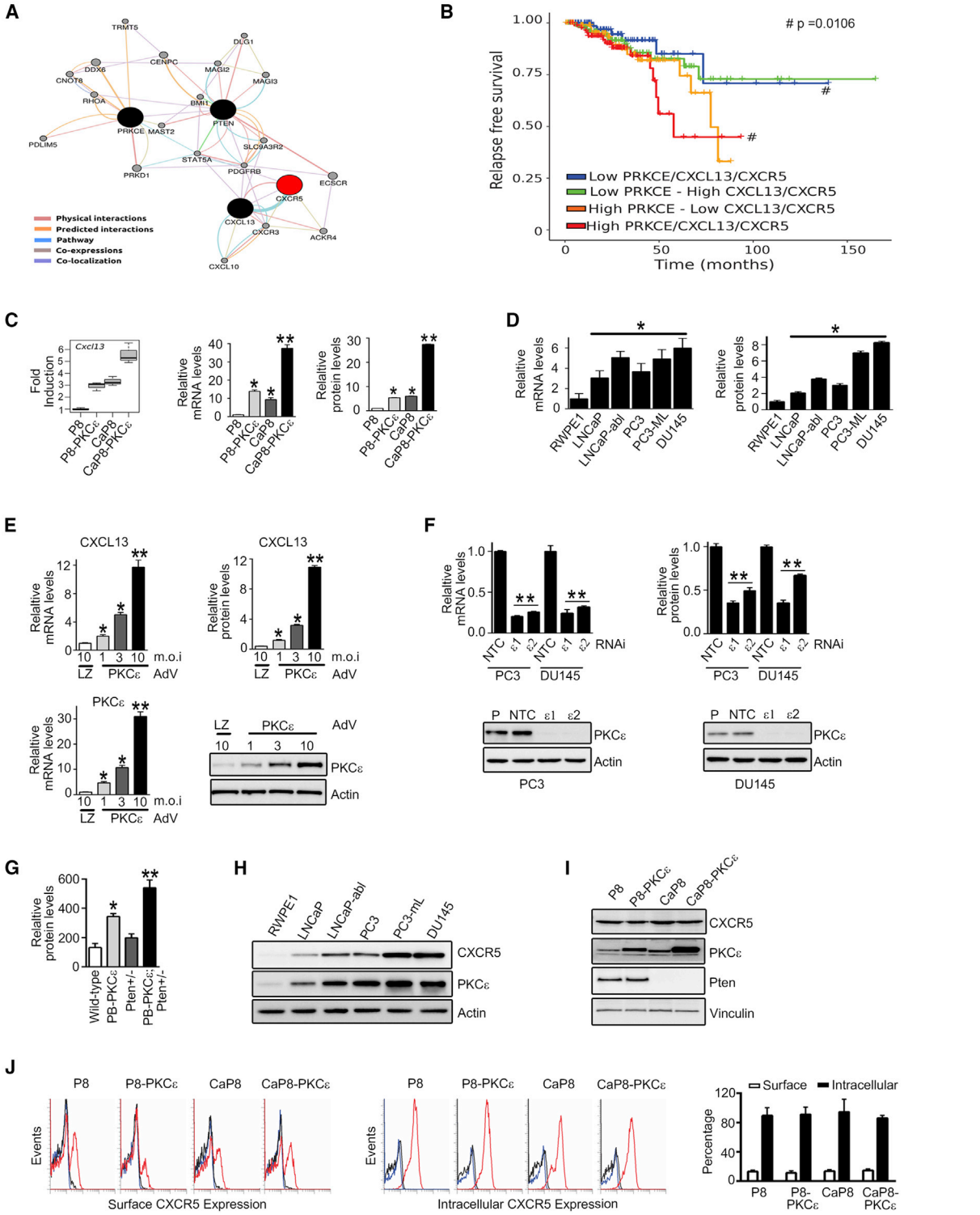


Figure 4. Association between PKCε and CXCL13 in Prostate Cancer

(A) Functional association among PKCε, Pten, CXCL13, and CXCR5 based on co-expression, physical interaction, pathways and co-localization data, using GeneMania.

(B) Kaplan-Meier analysis was performed among 365 patients with prostate carcinomas obtained from the TCGA-PRAD project. Patients were grouped as 82 with low PRKCE/CXCL13/CXCR5, 107 with low PRKCE-high CXCL13/CXCR5, 92 with high PRKCE-low CXCL13/CXCR5, and 84 with high PRKCE/CXCL13/CXCR5. p value is reported for curves (marked with #).

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this cell line and primary prostate carcinomas and/or metastatic samples, as shown in the Euler diagram in Figure 3F (see also Figure 3A; list of genes in Table S2). Among these genes, 140 were associated with prostate tumor metastases, 35 with primary tumors, and 14 with primary and metastatic prostate samples. Thus, striking similarities in the pattern of gene expression exist between the murine CaP8-PKCε genotype and that observed in human prostate cancer metastasis (Figure S4D).

PKCε Regulates CXCL13 Expression in Prostate Cancer Cells

As indicated above, the top hit from our microarray analysis in CaP8-PKCε cells was *CXCL13*. The chemokine CXCL13 (originally known as B cell chemoattractant, BCL) and its receptor, the GPCR C-X-C motif receptor 5 (CXCR5), emerged as pivotal players in the progression of many cancers (Airoldi et al., 2008; Biswas et al., 2014; Sambandam et al., 2013), including prostate cancer (Ammirante et al., 2014; El Haibi et al., 2010; Singh et al., 2009a, 2009b). Enhanced CXCR5 expression and hyperactivation of CXCR5 effectors occur in prostate cancer, and serum CXCL13 levels have been postulated as a biomarker of prostate cancer progression (Singh et al., 2009a, 2009b). A significant correlation between CXCL13 and CXCR5 expression occurs in human prostate cancer (Figure S4E). Further, a pathway reconstruction approach using GeneMania revealed *CXCR5* as a gene functionally related with *PRKCE*, *PTEN*, and *CXCL13* based on co-expression and physical interaction (Figure 4A). Kaplan-Meier analysis revealed the shortest recurrence-free survival specifically in patients categorized as high *PRKCE*/high *CXCL13*-*CXCR5* (Figure 4B).

As expected from the microarray data (Figure 4C, left panel), qPCR validation showed a clear elevation in *Cxcl13* mRNA levels in P8-PKCε and CaP8 cells relative to P8 cells (13- and 9-fold, respectively) and a striking ~40-fold upregulation in CaP8-PKCε cells (Figure 4C, middle panel). When CXCL13 protein levels were determined in the culture media by ELISA, a similar trend was observed (Figure 4C, right panel). Upregulation of *CXCL13* mRNA levels was found in established human prostate cancer cells relative to non-transformed RWPE-1 cells (Figure 4D, left panel). Accordingly, CXCL13 protein release was higher in prostate cancer cell lines, particularly in aggressive androgen-independent cell lines (Figure 4D, right panel), which also express high PKCε levels (Benavides et al., 2011; Garg et al., 2012).

A causal relationship between PKCε levels and CXCL13 induction was observed in P8 cells in which PKCε was overexpressed using an adenovirus (AdV) (Figure 4E). In addition, silencing PKCε expression from PC3 and DU145 prostate cancer cells considerably reduced CXCL13 mRNA and secreted protein levels (Figure 4F). Interestingly, we observed a significant elevation in serum CXCL13 levels in PKCε transgenic mice, particularly in PB-PKCε;Pten$^{+/-}$ mice (Figure 4G). Together, these observations argue for a stringent regulation of CXCL13 expression by PKCε in prostate cancer.

CXCR5, the CXCL13 receptor, is overexpressed in prostate tumors (Singh et al., 2009b). Consistent with this, prostate cancer cell lines display higher CXCR5 levels than RWPE1 cells (Figure 4H). However, we could not find appreciable differences in CXCR5 expression among P8, P8-PKCε, CaP8, and CaP8-PKCε cells (Figure 4I). Considering that each cell line is exposed to different CXCL13 levels in the medium, which may lead to receptor internalization, we next asked if changes in surface CXCR5 expression occur. Still, there were no significant differences among the murine cell lines, both in surface and total CXCR5 receptor levels (Figure 4J). Therefore, although PKCε overexpression and Pten loss are causally linked to the CXCL13 induction, they do not seem to influence CXCR5 expression.

An Autocrine CXCL13-CXCR5 Loop Mediates Migration Driven by PKCε Overexpression and Pten Loss

Next, we wished to test if the enhanced production of CXCL13 contributes to PKCε-driven phenotypes. We first assessed the activity of conditioned media (CM) collected from PKCε-overexpressing and/or Pten-depleted prostate epithelial cells. CXCL13 levels in the medium peak at 16 hr following cell seeding (Figure S5A). Conditioned media from P8, P8-PKCε, CaP8, and CaP8-PKCε cells was therefore collected at 16 hr and assessed for their "pro-migratory" activity when added to naive P8 cells. The ranking of activity of the different conditioned media was CM-CaP8-PKCε > CM-P8-PKCε > CM-CaP8 > CM-P8 cells (Figure 5A), which is in concurrence with the gradation of CXCL13 production from each cell line (see Figure 4C).

(C) CXCL13 expression in prostate epithelial cell lines. Left: CXCL13 mRNA expression from microarray data. Middle: CXCL13 mRNA expression by qPCR. Right: CXCL13 protein levels in the culture media by ELISA. Results normalized to P8 cells are expressed as mean ± SD of triplicate measurements. For qPCR and ELISA, two experiments gave similar results.

(D) Left: CXCL13 mRNA levels in human prostate cancer cells. Right: CXCL13 protein levels in culture media. Results normalized to RWPE-1 cells are expressed as mean ± SD of triplicate measurements.

(E) P8 cells were infected with different MOIs of PKCε or LacZ AdV (*LZ*, control) for 24 hr. Top left: CXCL13 mRNA levels by qPCR. Top right: CXCL13 protein levels in culture media assessed by ELISA 16 hr after infection. Bottom: PKCε levels determined by qPCR and western blot. Results were expressed as fold-increase relative to *LZ*.

(F) PC3 and DU145 cells were transfected with RNAi duplexes for PKCε (ε1 and ε2) or a non-target control (NTC) RNAi. *P*, parental cells. Left: CXCL13 mRNA levels determined by qPCR. Right: CXCL13 protein levels in culture media assessed by ELISA 16 hr after transfection. Western blot for PKCε expression is shown. Results are normalized to NTC transfected cells.

(G) CXCL13 serum levels in mice by ELISA.

(H) CXCR5 expression in human prostate cancer cells by western blot.

(I) CXCR5 expression in murine cells by western blot.

(J) CXCR5 levels in murine cells by flow cytometry. Left and middle: representative experiments. Right: quantification expressed as mean ± SD.

In (C)–(F) *$p < 0.05$ and **$p < 0.01$.

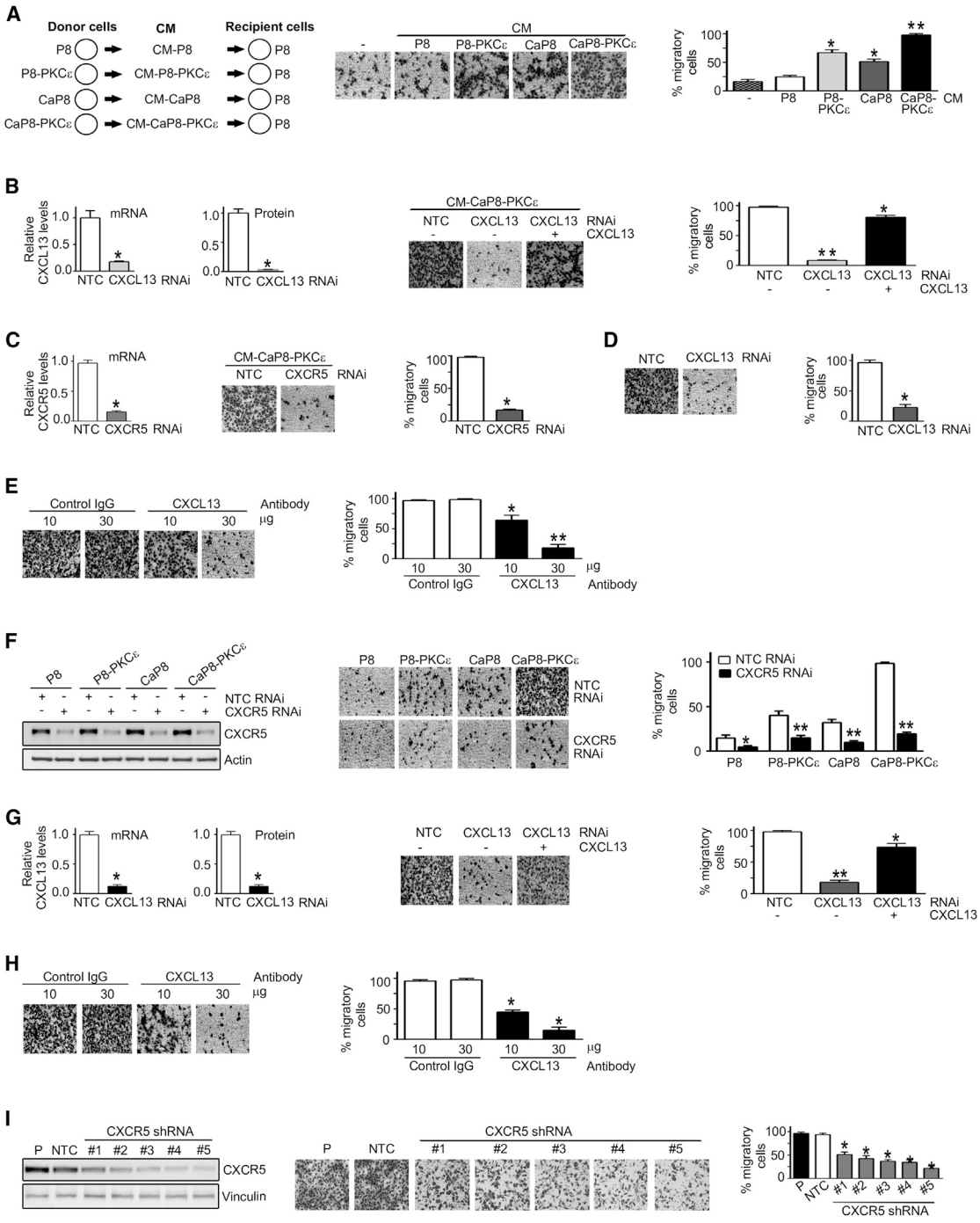

Figure 5. PKCε Overexpression and Pten Loss Induce Migration through an Autocrine CXCL13-CXCR5 Loop

(A) P8 cells were treated with conditioned medium from P8, P8-PKCε, CaP8, or CaP8-PKCε cells and migration assessed using a Boyden chamber. Left: experimental scheme. Middle: representative images. Right: quantification of migrating cells by contrast microscopy in five independent fields. Results are expressed as mean ± SD of triplicate measurements. Two experiments gave similar results.

(B) Conditioned medium was collected from CaP8-PKCε cells subject to either CXCL13 or non-target control (*NTC*) RNAi. Left: CXCL13 mRNA levels in CaP8-PKCε cells (qPCR) and protein levels in CM-CaP8-PKCε cells (ELISA). Results normalized to NTC were expressed as mean ± SD (n = 3). Middle: P8 cell migration was determined after treatment with conditioned medium from CaP8-PKCε cells subject to either CXCL13 or NTC RNAi. Reconstitution with recombinant CXCL13 (100 ng/mL) added to the conditioned medium was done where indicated. Right: quantification of migrating cells.

(C) P8 cells subjected to either CXCR5 or NTC RNAi were treated with conditioned medium collected from CaP8-PKCε cells. Left: CXCR5 mRNA levels in P8 (recipient) cells were determined by qPCR. Results normalized to NTC were expressed as mean ± SD (n = 3). Middle: representative images. Right: quantification of migrating cells.

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To determine if this pro-migratory effect is causally related to the released CXCL13, CaP8-PKCε cells were subject to CXCL13 RNAi depletion, which as expected reduced CXCL13 mRNA levels and protein release (Figure 5B, left panel, and Figure S5B). Notably, conditioned medium from CXCL13-depleted CaP8-PKCε cells significantly lost its ability to induce a migratory response when added to naive P8, P8-PKCε, or CaP8 cells. This effect was rescued by exogenous addition of CXCL13 (Figure 5B, middle and right panels, and Figures S5C–S5E). In addition, when CXCR5 expression in the recipient naive P8 cells was silenced, the pro-migratory activity of CM-CaP8-PKCε was essentially lost (Figures 5C and S5F), thus hinting at the important role of CXCL13 in this context.

To confirm the functional relevance of the enhanced CXCL13 production via an autocrine loop, we used three additional approaches. First, CXCL13 RNAi abrogated the migratory activity of CaP8-PKCε cells (Figures 5D and S5G). Second, a neutralizing anti-murine CXCL13 antibody dose-dependently inhibited migration of CaP8-PKCε cells compared to a non-specific isotype control antibody (Figure 5E). Lastly, silencing CXCR5 expression from PKCε-overexpressing and/or Pten-depleted cells markedly inhibited their migratory activities (Figure 5F). Similar experiments in human PC3 cells showed that CXCL13 RNAi, which depleted CXCL13 mRNA levels and protein release, markedly impaired migration, an effect rescued by the addition of recombinant CXCL13 (Figure 5G). Similarly, PC3 cell migration was inhibited by the addition of a neutralizing anti-human CXCL13 antibody (Figure 5H) or CXCR5 silencing (Figure 5I). Taken together, these results demonstrate a fundamental role of the CXCL13-CXCR5 autocrine axis in driving prostate cancer cell migration.

The CXCL13-CXCR5 Axis Contributes to the Growth and Tumorigenicity of Prostate Cancer Cells

Next, we aimed to determine if the CXCL13-CXCR5 pathway was involved in growth driven by PKCε overexpression. To address this issue, we first used CXCR5-depleted murine cell lines (shown in Figure 5F). CaP8-PKCε cells, which display the highest growth in culture (see Figures 2 and S2), significantly reduced their proliferative capacity upon CXCR5 RNAi depletion. A comparable inhibitory effect of CXCR5 RNAi was observed in P8-PKCε and CaP8 cells (Figure 6A). Stable CXCR5 and CXCL13 depletion from CaP8-PKCε cells using small hairpin RNA (shRNA) lentiviruses also impaired their ability to form colonies in soft agar (Figures 6B and S6) as well as tumorigenic capacity in nude mice (Figure 6C).

Next, we extended our growth and tumorigenicity studies to human PC3 cells subject to stable CXCR5 RNAi depletion (see Figure 5I). We found that CXCR5 was required for the growth of PC3 cells in soft agar and nude mice (Figures 6D and 6E). Altogether, our findings underscore the requirement of the autocrine CXCR5-CXCL13 axis in growth and tumorigenic activity of prostate cancer cells.

CXCL13 Upregulation Is Mediated by the Non-canonical NF-κB Pathway

To dissect the mechanisms governing CXCL13 upregulation by PKCε overexpression and Pten loss, we first used inhibitors of various signaling pathways. As expected, the PKC inhibitor GF109203X and the PI3K inhibitor BKM120 significantly reduced CXCL13 mRNA levels and protein release from CaP8-PKCε and PC3 cells. BX795, an inhibitor of PDK1, a PI3K-dependent kinase acting upstream of PKCs, had no effect on CXCL13 levels. Interestingly, CXCL13 expression and release were abrogated by NF-κB inhibitors IκB kinase (IKK)16 and wedelolactone (Figures 7A and S7).

PKCε activation significantly impacts the transcriptional regulation of genes (Garg et al., 2013). We therefore focused on transcriptional activation of the CXCL13 gene using a CXCL13 promoter luciferase reporter assay. Notably, P8-PKCε and CaP8 cells display enhanced CXCL13 reporter activity relative to P8 cells. This effect was even larger in CaP8-PKCε cells (Figure 7B). Interestingly, the CXCL13 reporter activity in CaP8-PKCε cells was sensitive to PKC, PI3K, and NF-κB, but not PDK1, inhibition (Figure 7C).

NF-κB is a known effector of PKCε and PI3K pathways (Fernandez-Marcos et al., 2009; Garg et al., 2012). Silencing key elements of the NF-κB pathway, IKKα and IKKβ, in CaP8-PKCε cells led to ~84% and ~31% inhibition in CXCL13 mRNA levels, respectively. As IKKα signals preferentially toward the non-canonical NF-κB pathway, we assessed the effect of knocking down NF-κB inducing kinase (NIK), a kinase associated with IKKα in this cascade. Remarkably, similar to IKKα RNAi, NIK RNAi abolished CXCL13 mRNA expression as well as transcriptional activation of the CXCL13 gene (Figures 7D and 7E). In silico analysis of the CXCL13 promoter identified a putative non-canonical NF-κB site that has also been previously characterized (Bonizzi et al., 2004) as well as putative sites for previously described responsive elements regulated by PKCε, specifically octamer-binding transcription factor 1 (OCT1) and hepatocyte nuclear factor 1 (HNF-1) (Garg et al., 2013). Upon

(D) Migration of CaP8-PKCε cells subject to either CXCL13 or NTC RNAi. Left: representative images. Right: quantification of migrating cells.
(E) Migration of CaP8-PKCε cells incubated with either a neutralizing anti-murine CXCL13 or a non-specific isotype control antibody. Left panel: representative images. Right panel: quantification of migrating cells.
(F) P8, P8-PKCε, CaP8, and CaP8-PKCε cells were infected with either CXCR5 or NTC shRNA lentiviruses, selected with puromycin and their migration assessed in a Boyden chamber. Left: CXCR5 expression by western blot. Middle: representative images. Right: quantification of migrating cells.
(G) Effect of CXCL13 or NTC RNAi on PC3 cell migration. Left: CXCL13 mRNA levels (qPCR) and protein release (ELISA) were determined. Results normalized to NTC were expressed as mean ± SD (n = 3). Middle: representative experiment. Reconstitution with recombinant CXCL13 (100 ng/mL) is shown. Right: quantification of migrating cells.
(H) Migration of PC3 cells incubated with either a neutralizing anti-human CXCL13 or a non-specific isotype control antibody. Left: representative images. Right: quantification of migrating cells.
(I) PC3 cells were infected with different CXCR5 or NTC shRNA lentiviruses, followed by puromycin selection. Left: western blot for CXCR5 expression. Middle: representative images. Right: quantification of migrating cells.
In (A)–(I) *$p < 0.05$ and **$p < 0.01$.

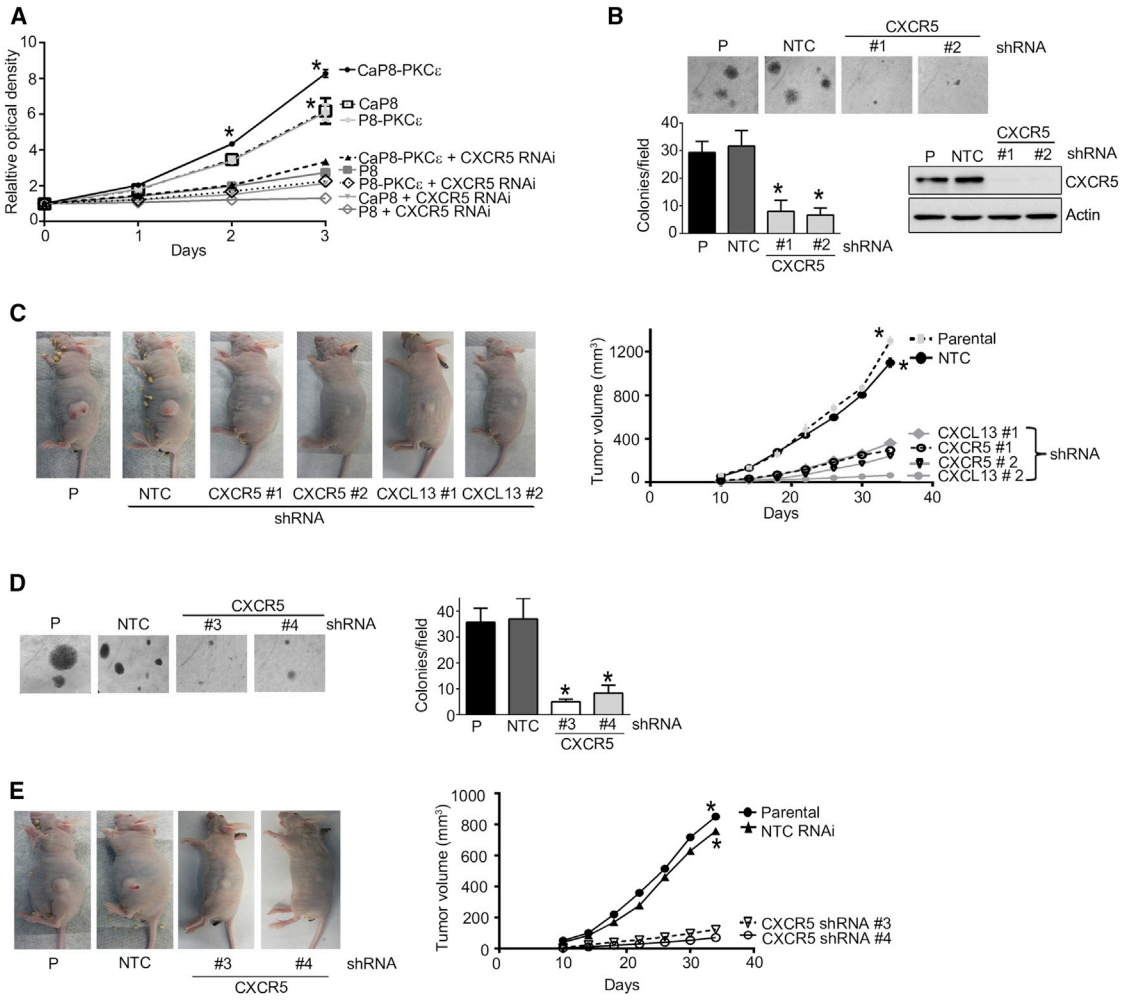

Figure 6. The CXCL13-CXCR5 Axis Mediates Tumorigenesis of Prostate Cancer Cells

(A) Proliferation of P8, P8-PKCε, CaP8, and CaP8-PKCε cells infected with either CXCR5 or NTC shRNA lentiviruses.

(B) Anchorage-independent growth in soft agar. Top: representative experiment. Bottom left: quantification of colonies/field. Bottom right: western blot for CXCR5 expression.

(C) CaP8-PKCε cells were infected with CXCR5, CXCL13 or NTC shRNA lentiviruses and selected with puromycin. Left: representative pictures of nude mice 35 days after s.c. inoculation. Right: tumor volume, expressed as mean ± SD (n = 5 mice/group).

(D) Growth in soft agar of CXCR5 stably depleted PC3 cells. Left: representative experiment. Right: quantification of colonies per field.

(E) Effect of CXCR5 silencing on PC3 tumor growth. Left: representative pictures of nude mice (day 35). Right: tumor volume, expressed as mean ± SD (n = 5 mice/group).

In (A)–(E) *p < 0.05.

mutation of each of these elements, we observed that only the NF-κB responsive element was required for CXCL13 reporter activity in CaP8-PKCε cells (Figure 7F). Overall, these results establish a prominent role for the non-canonical NF-κB pathway in the upregulation of CXCL13 driven by PKCε overexpression and Pten loss via a transcriptional mechanism.

DISCUSSION

Cooperativity between PKCε Overexpression and Pten Deficiency in Prostate Cancer

Our study provides evidence that PKCε overexpression contributes in conjunction with Pten deficiency to the development of prostate cancer. PKCε overexpression is a signature of many epithelial cancers and has been widely associated with cancer progression, including prostate cancer (Aziz et al., 2007; Garg et al., 2014). Prostate-specific transgenic overexpression of PKCε in mice confers a preneoplastic phenotype and hyperactivation of pro-survival pathways (Benavides et al., 2011; Garg et al., 2014). The appearance of invasive prostatic ACs in PB-PKCε;Pten$^{+/-}$ compound mice underscores the cooperative effect of PKCε with other common disease alterations (Blando et al., 2011; Carver et al., 2009; Zhong et al., 2006). Pten loss leads to PI3K/Akt activation and castration-resistant growth in mice (Mulholland et al., 2011). Likewise, mouse transgenic overexpression of PKCε protects against apoptosis in response to

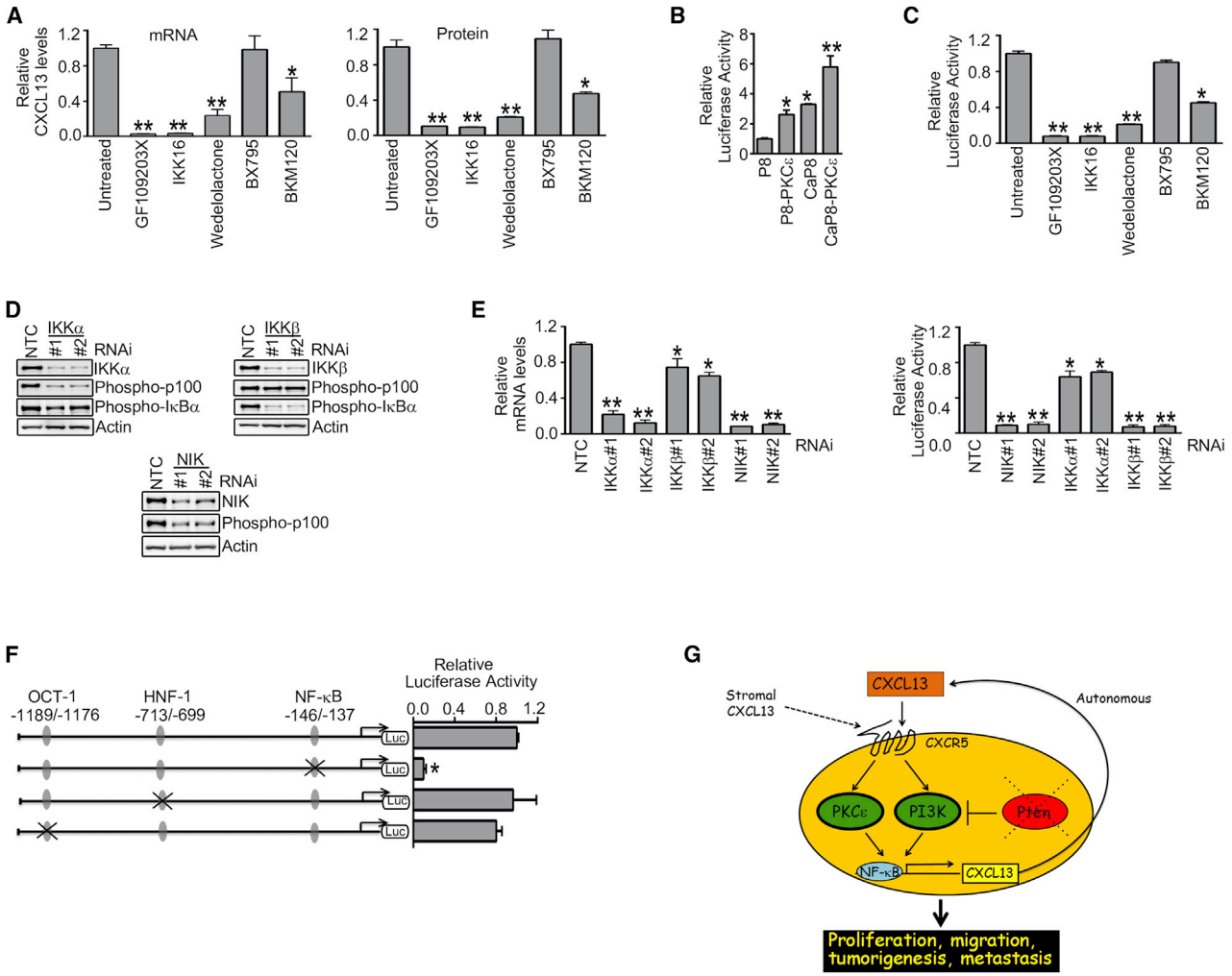

Figure 7. The NF-κB Non-canonical Pathway Mediates Transcriptional Activation of the CXCL13 Promoter

(A) Effect of GFX (0.5 μM), IKK16 (5 μM), wedelolactone (10 μM), BX795 (5 μM), and BKM120 (0.3 μM) on CXCL13 expression in CaP8-PKCε cells. Left: mRNA levels. Right: protein levels in the culture media.

(B) CXCL13 promoter luciferase reporter activity in murine prostate cells.

(C) Effect of inhibitors on CXCL13 promoter activity in CaP8-PKCε cells.

(D) IKKα, IKKβ, and NIK RNAi depletion by western blot.

(E) Effect of IKKα, IKKβ, and NIK RNAi depletion. Left: CXCL13 mRNA expression. Right: CXCL13 promoter activity.

(F) Effect of mutations in responsive elements on CXCL13 promoter activity.

(G) Model: the CXCL13-CXCR5 axis as an effector of PKCε overexpression and Pten loss.

In (A)–(C), (E), and (F), *$p < 0.05$ and **$p < 0.01$.

androgen ablation, which is consistent with previously established PKCε roles in the activation of pro-survival pathways (Aziz et al., 2007; Garg et al., 2014; Griner and Kazanietz, 2007; Jain and Basu, 2014). PKCε overexpression also contributes to tumorigenesis by conferring enhanced growth via Erk (Benavides et al., 2011), an effect that is potentiated in the context of PTEN deficiency. The aggressiveness of the prostate ACs originated in PB-PKCε;Pten$^{+/-}$ mice may highlight additional functions, such as genomic instability and accumulation of secondary mutations common upon Pten loss (Hubbard et al., 2016) or facilitation of EMT due to PKCε overexpression (Jain and Basu, 2014).

Despite the similar gross pathology of prostatic lesions from PB-PKCε and Pten$^{+/-}$ mice, our microarray analysis revealed both common and distinctive genetic signatures for PKCε overexpression and/or Pten deficiency. A similar scenario has been described upon comparison of PIN lesions induced by p110β, activated Akt and Pten deficiency (Lee et al., 2010). Most remarkably, as revealed by our GO analysis, cells displaying both PKCε overexpression and Pten loss, which acquire tumorigenic and highly migratory/invasive properties, are selectively enriched in a number of processes related to tumorigenesis and metastasis. Crucial roles for PKCε have been established in cancer cell migration and invasion. For

example, PKCε-depleted lung cancer cells have impaired Rac-dependent cell motility, invasion, and metastatic capacity (Caino et al., 2012b). PKCε promotes the secretion of pro-invasive factors and matrix metalloproteases (MMPs) and has been implicated in the formation of invadopodial-like structures (Gutierrez-Uzquiza et al., 2015; Tachado et al., 2002). One scenario currently under investigation in our laboratory is that Pten loss leads to a hyperactivated PKCε status, as indicated by the enhanced membrane-associated PKCε in Pten-depleted cells (data not shown). Not surprisingly, expression of a constitutively active PKCε mutant enhances growth and invasiveness (Garczarczyk et al., 2009). A PKCε hyperactive status is likely the result of excessive inputs, ultimately contributing to the tumorigenic and metastatic phenotype, which seem to be independent of the upstream PKC kinase PDK1.

The CXCL13-CXCR5 Axis as a Mediator of PKCε-Driven Phenotypes

We identified *Cxcl13* as a gene induced by PKCε overexpression and *Pten* loss in a cooperative manner. Emerging information assigned crucial roles to the CXCL13-CXCR5 axis in the progression of various epithelial cancers, and studies found that CXCL13 can be produced by tumor cells (Airoldi et al., 2008; Ammirante et al., 2014; Biswas et al., 2014; Gaulard and de Leval, 2011; Sambandam et al., 2013). Interestingly, serum CXCL13 levels positively correlate with prostate cancer progression in patients, and we found accordingly elevated serum CXCL13 levels in PB-PKCε;Pten$^{+/-}$ mice. CXCR5 signals through Erk, PI3K, and Rac in prostate cancer cells leading to proliferative and migratory/invasive phenotypes and couples to Gα subunits that activate phospholipase C, the enzyme responsible for DAG generation, the endogenous activator of PKCε (El Haibi et al., 2010, 2011, 2013; Singh et al., 2009a, 2009b). PKCε and PI3K activation due to Pten loss promotes CXCL13 production and release, thus contributing to CXCR5 signal amplification and ultimately resulting in an autocrine tumorigenic/metastatic vicious cycle. Consistent with this model (Figure 7G), the proliferative and motile capacities of CaP8-PKCε and PC3 prostate cancer cells, which express high PKCε levels and are Pten deficient, are impaired upon disruption of the CXCL13-CXCR5 axis.

One interesting observation in this study is the identification of the non-canonical NF-κB pathway as a converging point for signals from PKCε overexpression and Pten loss. Indeed, interfering with NIK-IKKα impaired CXCL13 induction. Based on the established role of this pathway in transcriptional activation and its proposed relevance in prostate cancer (Garg et al., 2014; Holley et al., 2010; Lessard et al., 2005), we focused our attention on the CXCL13 promoter, which contains a well-defined binding site for RelB/p52 (Bonizzi et al., 2004). Notably, disruption of this responsive element in the CXCL13 promoter reduced transcriptional activation in cells subject to PKCε overexpression and Pten loss. We also found that mitogen-activated protein kinase kinase (MEK) and Akt inhibitors reduce CXCL13 expression and transcriptional activation (data not shown), suggesting the possibility of these kinases as required downstream effectors or acting as parallel mechanisms. It is worth mentioning that the canonical NF-κB pathway has been implicated in prostate cancer progression driven by PTEN and Par4 loss (Fernandez-Marcos et al., 2009). Data from our laboratory also suggest that PKCε activates genes regulated by the canonical NF-κB pathway (Garg et al., 2012), also in cooperation with Pten loss (data not shown).

In addition to the cell-autonomous autocrine loop described above, stromal cells in prostate tumors, specifically cancer-associated myofibroblasts, produce CXCL13, leading to B cell recruitment and castration-resistant prostate cancer (Ammirante et al., 2014). Therefore, CXCL13 produced by both cancer and stromal cells creates a pro-tumorigenic environment. An additional consideration is that CXCR5, which is elevated in prostate cancer cells, may be implicated in site-specific metastasis. Conceivably, CXCL13 produced in the bone microenvironment may contribute to attracting prostate cancer cells to this preferred site for prostate cancer cell metastasis (Sambandam et al., 2013). CXCL13 is known to increase the expression of various mesenchymal markers and MMPs, therefore enhancing invasive capacity, and the expression of receptor activator of nuclear factor kappa-B ligand (RANKL), a driver for castration-resistant prostate cancer and skeletal metastasis (Biswas et al., 2014; Sambandam et al., 2013). Therefore, an attractive possibility is that targeting the CXCL13-CXCR5 pathway could represent a beneficial therapeutic approach for the treatment of advanced prostate cancer patients.

EXPERIMENTAL PROCEDURES

Reagents

CXCL13, IGF-1, and PDGF were purchased from R&D Systems. EGF was procured from BD Biosciences. Blasticidin and bovine pituitary extract were obtained from Life Technologies. Insulin was purchased from Sigma-Aldrich.

Cell Culture and Western Blot

Culture of human prostate cells and western blot are described elsewhere (Benavides et al., 2011; Garg et al., 2012). For more information about the antibodies used, see Supplemental Experimental Procedures. Murine P8/CaP8 prostate epithelial cells were kindly provided by Dr. Hong Wu (UCLA) (Jiao et al., 2007).

Generation of Mouse Models, Histopathology, and Immunohistochemistry

The generation of PB-PKCε transgenic mice in pure FVB/N background (co-isogenic line) is described elsewhere (Benavides et al., 2011). Crossing of PB-PKCε with *Pten*$^{+/-}$ mice, phenotypic analysis, and immunohistochemistry (IHC) procedures are described in Supplemental Experimental Procedures.

qPCR, RNAi, Flow Cytometry, Generation of PKCε-Overexpressing Cells, and CXCL13 Promoter Analysis

Detailed procedures are described in Supplemental Experimental Procedures.

CXCL13 ELISA Measurements

Cells (1–3 × 10^5 cells/well) were seeded in six-well plates, and conditioned medium was collected at different times (0–24 hr). CXCL13 levels were measured using either human or mouse CXCL13 ELISA kits (R&D Systems).

Adenoviral Infections

Infections with PKCε or LacZ (control) AdVs were done as previously described (Benavides et al., 2011; Meshki et al., 2010).

Cell Proliferation, Colony Formation, Migration, and In Vivo Tumorigenesis Assays

These assays are described elsewhere (Caino et al., 2012a, 2012b). For detailed information, see Supplemental Experimental Procedures.

Microarray and Data Mining Analysis

Total RNA was obtained using the miRNeasy Mini Kit (QIAGEN). Detailed information on microarrays, software, and databases used for bioinformatics are presented in Supplemental Experimental Procedures.

Statistical Analysis

Data were analyzed using either a Student's t test or ANOVA. $p < 0.05$ was considered statistically significant.

ACCESSION NUMBERS

The accession number for the raw and preprocessed datasets reported in this paper is GEO: GSE86257.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.03.042.

AUTHOR CONTRIBUTIONS

R.G. performed all cell culture, nude mice, and microarray studies. C.J.P., F.B., and R.G. performed experiments with transgenic and KO mice, including generation of mouse models. J.M.B. and F.B. carried out pathological analysis of mouse prostates and IHC experiments. M.C.A. and R.G. performed the analysis of microarray data and bioinformatics. R.G., J.M.B., C.J.P., M.C.A., F.B., and M.G.K. provided insightful discussions, designed experiments, and analyzed data. R.G., J.M.B., M.C.A., F.B., and M.G.K. wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by NIH grants R01-CA089202, R01-CA189765, and R01-CA196232 and DoD grant PC130641 (to M.G.K.), FONCYT (Argentina) grant PICT 0275 (to M.C.A.), and DoD grant W81XWH-12-1-0009 (to R.G.). This study made use of the Research Animal Support Facility-Smithville, including Laboratory Animal Genetic Services and Mutant Mouse Pathology Services, which are supported by DHHS/NCI Cancer Center support grant P30 CA016672.

Received: October 10, 2016
Revised: February 1, 2017
Accepted: March 13, 2017
Published: April 11, 2017

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