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Tumor and Stem Cell Biology

Pten Loss and RAS/MAPK Activation Cooperate to Promote EMT and Metastasis Initiated from Prostate Cancer Stem/Progenitor Cells

David J. Mulholland1, Naoko Kobayashi1, Marcus Ruscetti1, Allen Zhi1, Linh M. Tran1, Jiaoti Huang2, Martin Gleave4, and Hong Wu1,3

Abstract

PTEN loss or PI3K/AKT signaling pathway activation correlates with human prostate cancer progression and metastasis. However, in preclinical murine models, deletion of Pten alone fails to mimic the significant metastatic burden that frequently accompanies the end stage of human disease. To identify additional pathway alterations that cooperate with PTEN loss in prostate cancer progression, we surveyed human prostate cancer tissue microarrays and found that the RAS/MAPK pathway is significantly elevated in both primary and metastatic lesions. In an attempt to model this event, we crossed conditional activatable K-rasG12D/WT mice with the prostate conditional Pten deletion model. Although RAS activation alone cannot initiate prostate cancer development, it significantly accelerated progression caused by PTEN loss, accompanied by epithelial-to-mesenchymal transition (EMT) and macrometastasis with 100% penetrance. A novel stem/progenitor subpopulation with mesenchymal characteristics was isolated from the compound mutant prostates, which was highly metastatic upon orthotopic transplantation. Importantly, inhibition of RAS/MAPK signaling by PD325901, a mitogen–activated protein (MAP)–extracellular signal–regulated (ER) kinase (MEK) inhibitor, significantly reduced the metastatic progression initiated from transplanted stem/progenitor cells. Collectively, our findings indicate that activation of RAS/MAPK signaling serves as a potentiating second hit to alteration of the PTEN/PI3K/AKT axis, and cotargeting both the pathways is highly effective in preventing the development of metastatic prostate cancers.

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Introduction

Prostate cancer is the most common male malignancy and a frequent cause of mortality in Western countries (1). During late-stage disease, oncogenic signaling pathways act collaboratively to promote metastasis and castration-resistant prostate cancer (CRPC) development. Alteration of the PTEN/PI3K/AKT pathway is well correlated with prostate cancer development with about 70% of late-stage samples showing PTEN loss or phosphoinositide 3-kinase (PI3K) activation (2). The Pten-null prostate cancer model mimics human disease, including hyperplasia, pancreatic intraepithelial neoplasia, and invasive carcinoma, with defined kinetics (3). However, inactivation of Pten alone (3–5) or in combination with homozygous deletion of p53 (6, 7) or Nkx3.1 (8) fails to recapitulate the critical aspect of end-stage human prostate cancer, that is, significant metastatic burden. Thus, identification of signaling mechanisms that collaborate with alteration of the PI3K pathway in promoting prostate cancer metastasis is critical for modeling late stage of human disease and for testing therapeutic strategies.

Despite the low frequencies of RAS mutations (9–12) and RAS fusion events (13), compelling evidence suggests that RAS/MAPK pathway activation plays a significant role in human prostate cancer progression, particularly, in metastasis and CRPC development. Enhanced RAS signaling can reduce dependency for androgens in the LNCaP prostate cancer cell line (14), whereas inhibition of RAS can restore hormone dependence in C42 cells, a line that is otherwise hormone independent (14, 15). Furthermore, patients who have failed hormone ablation therapy display augmentation of p-mitogen–activated protein kinase (MAPK) levels, a downstream target of RAS signaling (16). Finally, RAS activation in the DU145 human prostate cancer cell line can promote metastasis to the brain and bone (17). Despite these in vitro observations, it is unclear that (i) whether activation of the RAS/MAPK pathway is sufficient to initiate the full spectrum of prostate cancer development and (ii) whether the RAS/MAPK pathway...
can collaborate with the PTEN/Pi3K pathway in promoting metastasis and CRPC development.

We hypothesized that activating the RAS/MAPK pathway in conjunction with reduced Pten dosage may promote metastasis. To test this hypothesis, we incorporated the activating K-rasG12D/W allelic (18), as a means to activate the RAS/MAPK axis, to the Pten-null prostate cancer model that we generated previously (3). We report here the important collaborative effects of RAS/MAPK and Pten/Pi3K pathways in promoting prostate cancer metastasis and potential molecular mechanisms underlying such collaboration. Collectively, our results suggest that RAS/MAPK pathway activation may serve as a critical “second hit” to Pten/Pi3K/AKT pathway alterations to androgen-dependent prostate cancer and CRPC.

Methods

Human tissue microarray and bone metastasis samples

Human prostate cancer tissue microarrays (TMA) are composed of 194 patients and 388 cores (19). Histopathologic composition of the array is outlined in Supplementary Fig. S1. All bone metastasis are from patients with prostate cancer with abnormal bone scans.

Mouse strains, tissue collection and reconstitution

Mutant mice with prostate-specific deletion of Pten were generated as previously described under a mixed background (3). To generate the Pb-Cre;Ptenfl/fl;K-rasG12D/W or Pb-Cre;Ptenfl/fl;K-rasG12D/W mice, Pb-Cre;Ptenfl/fl male mice were bred with female Cre;Ptenfl/fl;K-rasG12D/W mice (20). To generate Pb-Cre;Ptenfl/fl;K-rasG12D/W;LSL-Rosa26-LacZ or Pb-Cre;Ptenfl/fl;K-rasG12D/W;LSL-Rosa26-Luc mice, Cre;Ptenfl/fl;K-rasG12D/W male mice were bred with female, Cre;Ptenfl/fl;LSL-Rosa26-LacZ (21) or Cre;Ptenfl/fl;LSL-Rosa26-Luc (LSL-Rosa26-Luc was obtained from National Cancer Institute eMICe Strain 01XAC). All animal housing, breeding, and surgical procedures were conducted under the regulation of the Division of Laboratory Animal Medicine at the University of California at Los Angeles (UCLA; Los Angeles, CA).

mRNA extraction and microarray hybridization

RNA was extracted from pooled lobes resected from mutant prostates. Microarrays were conducted in the UCLA Clinical Microarray Facility using Affymetrix mouse 430 2 arrays. In brief, total RNA was extracted using the miRNeasy Mini Kit (Qiagen). Array hybridization, washing, and scanning were carried out as per the manufacturer’s instructions. For genes represented by multiple probes, its expression was represented by the average of its probe expressions. Microarray data are available at the National Center for Biotechnology Information Gene Expression Omnibus (GSE34839).

Rank–rank analysis

In rank–rank geometric overlap analysis (RRHO), genes in human data sets derived from the studies of Lapointe and colleagues (22) and Taylor and colleagues (2) were ranked on the basis of their log-transformed P values of t test comparing between 2 subgroups/genotypes as previously described (23, 24).

Immunohistochemistry and LacZ detection

To detect LacZ+ cells, frozen sections were fixed in methanol, followed by X-gal staining (25) for 6 to 12 hours, and then counterstained with Fast Red. Immunohistochemistry was carried out as previously described (3, 26) using the following antibodies: Pten (Cell Signaling Technology; 9559), p-MAPK (Cell Signaling Technology; 4376), androgen receptor (Ar; Santa Cruz; sc-816), pan-cytokeratin (Sigma; C1801), E-cadherin (Cell Signaling Technology; 610181), vimentin (Abcam; ab39376), p-AKT (Cell Signaling Technology; 3878), Ki67 (Vector; VP-RM04), and p63 (BD Transduction; 559952).

FACS analysis and cell sorting

Cell isolation was carried out as previously described (26) using the following fluorescence-activated cell-sorting (FACS) antibodies: Sca1-PE (BD Pharm; 553336), CD49F-APE (Biolegend; 313610), EpCAM-APC/Cy7 (Biolegend; 118218), and CD24-PE/Cy7 (Bio-Rad) compared with the RPL13a RNA quantity for each cDNA sample as an endogenous control. Primers used for cell lineage marker expression were used as previously described (27) and epithelial-to-mesenchymal transition (EMT) markers as follows: Cdh1: F, CAGGTCTCCTTCATGGTTTG; R, CTTCCGAAAGAAGGCTGTCC; Fl, F, AGCTTGGAGAGCCACCTAC; R, ACGTAGGAGTCGCCACGAGC; FoxC2; F, AAGA-
TCACCGCTGAACCCGAC; C, RACCTTTCCAGAGACCTCTATG; Mmp2; F, CACTCTACAAAAAGACCTT; R, GAAACA-
CACGCTTCTTCCTTCT; N-cad; F, CAGGTTCCTCAGTGTGGTTGC; R, CTTCCGAAAGAAGGCTGTCC; RPL13a: F, TACCGTGTGAAAGGCTCATAC; R, ATCCATCCCAACCTT GGAG; Snail; F, AAGATGCACATCCGAGAAGC; R, ATCTCTCTCATCCGAGTTG; Twist: F, CGGCTGTCATGTGCAAGTG; R, CAGCTTGGCATTGGAAGCT; Vim; F, CGGTGTCGCCGAA-
GAATGG; R, CCATTTTCGTTCAAGGTCAAG; Zeb1; F, CATGTGACCTGTGTGACAAG, R, GCGGTGATTCATGT-
GZ; Zeb2; F, TAGGCGTCGAGAAGAATGG; R, GCCATCTCCTTCTCCAGT.

Cell line

The Pten−/−;K-rasG12D cell line was isolated from a 10-week C57BL/6;K-rasG12D/W mutant prostate through FACS sorting of Lin−EpCAM<sup>hi</sup>CD24<sup>hi</sup> cells. Cells were passaged in culture at least 10 times and PCR authenticated for Pten deletion and the presence of the K-ras<sup>G12D</sup> activating allele. Pten−/−;K-rasG12D cells were infected with lentivirus carrying GFP.
Orthotopic injections, bioluminescence imaging, and measurement of lung lesions

Prostate sphere cells or subpopulations of primary cancer cells were isolated from C:\Pten\L:\K-ras\L:LSI-Luc mutant prostates. Prostate orthotopic injections were carried out using approximately 2,000 cells in 50% Matrigel/media using a 10 μL Hamilton syringe (Microliter #701). Tumor development was then monitored using bioluminescence detection (Xenogen IVIS, Caliper Life Sciences). For measurement of lung lesions, accumulated lesion area per mouse was measured and then calculated as a percentage of total lung section area. Tibial, orthotopic injections to NOD-SCID; II.2ry\null recipients were carried out using a 10 μL Hamilton syringe (Microliter #701) to deliver no more than 1,000 Pten\null;K-ras\G12D cells injected in 10 μL volume of 50% Matrigel/media.

Drug treatment

NOD-SCID; II.2ry\null mice with various orthotopic transplantations were treated with rapamycin (4 mg/kg/d, intraperitoneally) and/or PD325901 (5 mg/kg/d, per os) daily for 14 days.

Results

RAS/MAPK pathway is activated in human primary and metastatic prostate cancer lesions

Of several well-known pathway alterations found in human prostate cancers is the RAS/RAF/MAPK pathway, showing frequencies of 43% and 90% alteration in primary and metastatic lesions, respectively (2). To investigate the potential collaboration between the PTEN/PI3K and RAS/MAPK pathways, we assessed the correlation between PTEN loss and MAPK activation using (i) a human prostate cancer TMA composed of 194 patients and 388 cores (Fig. 1A and Supplementary Fig. S1) and (ii) 30 human prostate cancer bone metastasis specimens obtained from 4 U.S. medical institutions (Fig. 1B). While p-MAPK levels were not significantly elevated in untreated specimens, levels were significantly increased in neoadjuvant-treated (neoadjuvant hormone therapy) and recurrent patients with CRPC as compared with benign prostate hyperplasia specimens, coinciding with at least a 1-fold reduction in PTEN expression (Fig. 1A, * P < 0.05; ** P < 0.005). In metastatic bone lesions, we observed elevated p-MAPK staining in 80% (24 of 30) of samples (Fig. 1B), a finding similar to what was reported previously for lymph node metastasis (28). In nearly all bone lesions, PTEN expression was low or negative (Fig. 1B, right). Interestingly, prominent p-MAPK expression was found near the basal compartment, corresponding to the potential transient amplifying stem/progenitor cell populations in human bone metastatic lesions (Fig. 1B, arrow). Collectively, these data indicate that the RAS/MAPK signaling pathway is highly active in human prostate cancer specifically in patients who have received anti-androgen therapy. These data also suggest that there may be selection for coinciding activation of PI3K/AKT and RAS/MAPK signaling in patients with late-stage disease.

PTEN loss and RAS activation cooperate in accelerating primary and metastatic prostate cancer progression

To assess the role of RAS pathway activation in promoting prostate cancer development and metastasis, we conditionally activated K-ras in the prostatic epithelium by crossing the K-ras\G12D\W (K-ras\L:\W; ref. 29) allele to the Pb-Cre line (C; ref. 30). While RAS activation was sufficient to enhance p-MAPK levels, it failed to promote significant cell proliferation, AKT activation, and prostate cancer development (Supplementary Fig. S2). Therefore, activation of the RAS pathway alone is not sufficient to induce prostate cancer. To assess RAS/MAPK activation as an additional hit to PTEN/PI3K/AKT pathway alteration in promoting prostate cancer progression, we crossed Pb-Cre\PTen\null mice (C;Pten\null) with K-ras\W mice to generate C;Pten\L:\W;K-ras\L:\W mutants. In comparison to C;Pten\null mutants, simultaneously deleting Pten and activating Kras led to early lethality (Fig. 2A, comparing red and green lines) with enhanced progression at both gross (Fig. 2B; arrows, anterior lobes; solid black arrowheads, dorsolateral lobes) and histologic levels (Fig. 2C). While pathology in C;Pten\null mutants was predominantly adenocarcinoma localized to the dorsolateral lobes, C;Pten\L:\W;K-ras\L:\W mutants showed invasive carcinoma both in the dorsolateral and anterior lobes as early as 4 weeks with poorly differentiated carcinoma occurring by 10 weeks (Fig. 2B and C).

Because partially reduced PTEN expression occurs frequently during human prostate cancer progression (Fig. 1B; refs. 31, 32), we then considered whether loss of a single Pten allele could also cooperate with RAS activation. While under the genetic background we studied, neither C;Pten\W or C;Pten\L:\W;Pten\null mice showed little evidence of cancer when older than 1 year (data not shown and Supplementary Fig. S2), C;Pten\W;K-ras\L:\W mice developed focal neoplastic expansions by 10 weeks, invasive carcinoma by 20 weeks (Supplementary Fig. S4A and S4B), and lethality around 40 weeks (Fig. 2A, orange line) accompanied by PTEN loss and p-AKT-S473 activation (Supplementary Fig. S4C).

Importantly, in contrast to micrometastasis seen in 15% to 30% of age- and genetic background–matched C;Pten\null mice (3), both C;Pten\L:\W;K-ras\L:\W and C;Pten\W;K-ras\L:\W mutants developed macrometastatic lesions in the lung and liver with 100% penetrance (Fig. 3A). Lesions were largely pan-cytokeratin positive (Fig. 3A) with activated MAPK and PTEN loss (Fig. 3B). Interestingly, AR expression was highly heterogeneous in lung lesions (Fig. 3A) and primary tumor samples from C;Pten\W;K-ras\L:\W mutants (Supplementary Fig. S3A). Moreover, gene set enrichment analysis (GSEA) showed that C;Pten\L:\W;K-ras\L:\W mutant prostate has reduced expression of AR target genes (Supplementary Fig. S3C), in comparison to C;Pten\null mutants, including Mme, Mmb, and Nks3.1 (Supplementary Fig. S3C).

Importantly, cells with genotype of Cre\null, Pten deletion and activated Kras were detected in 4 of 6 bone marrow flushes of C;Pten\L:\W;K-ras\L:\W or C;Pten\W;K-ras\L:\W mutants (Fig. 3C). However, because of early lethality of these animals, we did not observe overt metastasis in the bone at the histologic level or by bone imaging (data not shown). Collectively, our results indicate that the cooperation between PTEN loss and RAS...
activation yields significantly enhanced metastatic prostate cancer progression in these new murine models.

RAS activation promotes Pten-null epithelial cells to undergo EMT and acquisition of a human prostate cancer signature

A striking feature found in C\(^+\);Pten\(^L/L\);K-ras\(^L/W\) and C\(^+\);Pten\(^L/W\);K-ras\(^L/W\) prostates was the mesenchymal morphology associated with the aggressive behavior of cancer cells, similar to poorly differentiated human cancers (Supplementary Fig. S5). Similar to human prostate cancer, we observed a loss of p63 expression in the basal compartment of cancerous acini of C\(^+\);Pten\(^L/L\);K-ras\(^L/W\) mutants (Supplementary Fig. S5A). We also observed further reduction of p53 and p27 in C\(^+\);Pten\(^L/L\);K-ras\(^L/W\) mutants compared with those with Pten single deletion (Supplementary Fig. S5B). Consistent with EMT phenotype, we observed enhanced N-cadherin expression in transition regions, especially in poorly differentiated cancer, indicating that many of these cells displayed neuroendocrine expansion. Interestingly, synaptophysin-positive cells were generally rare in this new model (Supplementary Fig. S5C).

To assess the role of Ras activation in promoting EMT in Pten-null epithelium, we examined regions with morphologic transition using the epithelial (E-cadherin) and mesenchymal markers (vimentin; Fig. 4A). While adenocarcinoma and mesenchymal cancer regions showed distinct marker expression, transition regions showed coexpression of both epithelial and mesenchymal markers (Fig. 4A, yellow in overlay).

To ascertain that the observed mesenchymal pathology occurred as a result of a true EMT, and not expansion of the native stromal compartment, we crossed a Cre-activatable LacZ reporter line (LSL-Rosa26-LacZ; ref. 25) onto the...
These data indicate that RAS activation facilitates EMT of mesenchymal morphology (Fig. 4B, arrowheads in middle) cells from epithelial acini (Fig. 4B, arrows) to regions with LSL-Rosa26-LacZ prostates showed expansion of lineage tracing for those C/+;PtenL/L;LSL-Rosa26-LacZ epithelium (data not shown); however, C+;PtenL/L;K-rasL/W mutants showed an expansion of LacZ-positive cells from epithelial acini (Fig. 4B, arrows) to regions with mesenchymal morphology (Fig. 4B, arrowheads in middle). These data indicate that RAS activation facilitates EMT of Pten-null epithelial cells.

We then conducted unbiased microarray analysis on age- and genetic background–matched C+;PtenL/-;K-rasL/W and C+;PtenL/- prostate samples (n = 3; 10 weeks) and found that 370 and 336 genes were significantly up- and downregulated for more than 3-fold, respectively, due to RAS activation. Among those upregulated genes, 11 were EMT-associated genes (P = 1.7e-13, Fisher’s exact test; Fig. 4C). We further validated the array analysis by real-time PCR using independent prostate samples from C+;PtenL/-;K-rasL/W, C+;PtenL/-, and C+;PtenL/-;K-rasL/W mutants at 10 weeks of age. Prostates from C+;PtenL/-;K-rasL/W mutants showed significantly enhanced expression of EMT markers including Snail (Snai1), vimentin (Vim), Fibronectin (Fn1), MMP2 (Mmp2), Twist (Twist1), Zeb1 (Zeb1), and Foxc2 (Foxc2; Fig. 4D). Thus, at the gene expression level, C+;PtenL/-;K-rasL/W mutants display an EMT signature.

Because of the association of PTEN/Pi3K/AKT and RAS/RAF/MAPK pathway alterations in human prostate cancer progression, we hypothesized that murine prostate cancer with concomitant PTEN and RAS pathway alterations may closely resemble gene signatures of end-stage human prostate cancers. To test this hypothesis, we used RHRO analysis to compare the overlap of differentially expressed genes in human primary versus metastatic tumors from either the data sets in the study of Lapointe and colleagues (22) or Taylor and colleagues (2) with either Pb-C+;PtenL/- or Pb-C+;PtenL/-;K-rasL/W mutants. The heatmap signifies that the bottom left (blue circle) and top right (red circle) corners indicate that primary human tumors shared the greatest overlap with Pb-C+;PtenL/- primary tumors whereas the human metastatic data set overlapped more with the signature derived from Pb-C+;PtenL/-;K-rasL/W mutants (Supplementary Fig. S6A).

On the basis of previously published RAS signature gene sets (33), several gene sets were noted to be altered to a greater extent in PtenK-ras metastatic lesions, similar to that of human disease, as exemplified by the downregulation of fibroblast growth factor receptor 2 (FGFR2) expression and enhanced UBE2C expression, a ubiquitin-conjugating enzyme known to be overexpressed in human prostate cancer (Supplementary Fig. S6B; ref. 34). Together, these analyses provide strong support of our hypothesis that the PtenK-ras model closely mimics the biology of human prostate cancer, especially metastatic disease.

**Pten loss and Ras activation cooperate to enhance stem/progenitor activity**

Recent studies suggest that EMT is associated with the formation of breast cancer stem cells (35) and the progression of prostate cancer (19, 36). To test whether RAS activation induces EMT in Pten-null prostatic stem/progenitor cells and consequently promotes prostate cancer progression and metastasis, we characterized prostatic stem/progenitor cells in vivo sphere-forming analysis. Our previous study indicates that LSC<sup>ras</sup> (Lin− Sca1<sup>+</sup> CD49f<sup>+</sup>) stem/progenitor cells have high sphere-forming activity and are both necessary and sufficient for initiating Pten-null prostate cancers (26). Similar to Pten-null prostates, the compound PtenK-ras prostates showed significant expansion of the leukemia stem cell (LSC<sup>ras</sup>) subpopulation (Fig. 5A, left; ***, P < 0.01, n = 4) and further enhanced sphere-forming activity (Fig. 5A, right; ***, P < 0.01). However, different from Pten-null prostates, the LSC<sup>ras</sup> (Lin− Sca1<sup>+</sup> CD49f<sup>+</sup>) subpopulation isolated from the compound mutants had significantly enhanced sphere-forming activity in free-floating conditions (Fig. 5A, right; **, P <
0.05). To assess whether certain epithelial stem/progenitor cells have acquired mesenchymal characteristics and, therefore, reduced epithelial marker expression on the cell surface, we isolated Lin⁻/⁻EpCAM⁻/⁻CD24⁻/⁻ cells from the above mutants and found that only Lin⁻/⁻EpCAM⁻/⁻CD24⁻/⁻ cells (Fig. 5B, right FACS plot) from C⁺;Pten⁻/⁻;K-ras⁺/⁻ mutants had significant sphere-forming activity (P < 0.001; Fig. 5A, right). Using real-time PCR analysis, we affirmed that LSC/high and LSC/low subpopulations corresponded to the basal (Ar, p63, Ck14), and luminal cell populations (Ar, Ck8, Ck18, E-cadh, and Psca), respectively, whereas the EpCAM⁺/⁺CD24⁺/⁺ subpopulation corresponded to mesenchymal cells on the basis of the heightened gene expression of Ar, Mmp2, N-cadherin, Snail, Twist, vimentin, and Zeb2 (Fig. 5B, n = 4). Therefore, Pten loss and Ras activation collaborate in stem/progenitor expansion and Ras activation promotes EMT in Pten-null sphere-forming cells.

**Stem/progenitor cells with Pten loss and Ras activation can reconstitute EMT and macrometastatic prostate cancer**

Our previous study showed that the LSC/high subpopulation or its derived sphere cells isolated from Pten-null primary cancers could reconstitute adenocarcinoma when subject to the prostate regeneration assay (26). Because primary C⁺; Pten⁻/⁻;K-ras⁺/⁻ mutants develop macrometastasis, we then considered whether sphere cells derived from these mutants could also initiate a metastatic phenotype via orthotopic
transplantations, an assay thought to closely mimic the natural metastatic process (37). To test this, we dissociated passage 3 sphere cells from C\(^{+};K\text{-ras}\text{L/W}\); C\(^{+};\text{Pten}\text{L/L}\), and C\(^{+};\text{Pten}\text{L/L};K\text{-ras}\text{L/W}\) mice followed by orthotopic injection of approximately 2 \(\times\) 10\(^3\) cells to the proximal region of the anterior lobe of NOD;SCID;IL2rg-null mice. Genotypes of sphere cells were confirmed by PCR analysis, before transplantation, on individual P3 spheres (Supplementary Fig. S7A and data not shown).

Although C\(^{+};\text{Pten}\text{L/L}\) (data not shown) and C\(^{+};\text{Pten}\text{L/L};K\text{-ras}\text{L/W}\) sphere cells could initiate primary engraftments after 3 to 4 weeks, only recipient mice with C\(^{+};\text{Pten}\text{L/L};K\text{-ras}\text{L/W}\) sphere cells appeared morbid with poorly differentiated carcinoma (Fig. 6A, left). Extensive micro- and macrometastases was observed in the lymph nodes, lung, and liver of mice that received C\(^{+};\text{Pten}\text{L/L};K\text{-ras}\text{L/W}\) mutant prostates. The metastatic lesions maintained morphology similar to the primary cancers (data not shown). Importantly, recipients of either C\(^{+};\text{K-ras}\text{L/W}\) or C\(^{+};\text{Pten}\text{L/L}\) sphere cell transplants revealed no detectable macrometastasis or morbidity by 10 weeks posttransplantation (data not shown), suggesting that concomitant alteration of both PTEN/PI3K and RAS/MAPK pathways in stem/progenitor cells is required for the metastasis development in the orthotopic transplantation models.

**Figure 4.** Pten loss and Ras pathway activation propagate an EMT signature. A, histology (left, top) and immunostains [E-cadherin (E-cad), vimentin (Vim); bottom] showing regions of transition between epithelial and mesenchymal phenotypes. Low magnification bar, 500 \(\mu\)m; high magnification bar, 100 \(\mu\)m. B, lineage tracing using \(\beta\)-gal staining and the LSL-Rosa26-LacZ reporter in conjunction with the epithelial specific probasin promoter in C\(^{+};\text{Pten}\text{L/L};K\text{-ras}\text{L/W}\) mutants (10 weeks). Low magnification bar, 500 \(\mu\)m; high magnification bar, 200 \(\mu\)m. C, gene microarray analysis showing EMT pathway gene activity in between C\(^{+};\text{Pten}\text{L/L};K\text{-ras}\text{L/W}\) and C\(^{+};\text{Pten}\text{L/L}\) mutants. D, RT-PCR confirmation of EMT gene alterations in C\(^{+};\text{Pten}\text{L/L};K\text{-ras}\text{L/W}\) mutant prostates (*, \(P < 0.05\)).
To further support our hypothesis that stem/progenitor cells can reconstitute both EMT and metastatic phenotypes, we FACS sorted the LSC\textsuperscript{high}, LSC\textsuperscript{low}, and EpCAM\textsuperscript{low}/CD24\textsuperscript{low} mesenchymal subpopulations from $C;Pten^{-/-};K-ras^{L/W}$, $C;Pten^{-/-}$, and $C;Pten^{-/-};K-ras^{L/W}$ mutant prostates at 8 to 10 weeks of age (Supplementary Fig. S7B and data not shown), followed by orthotopic transplantation. Consistent with our previous studies, transplantation of $Pten^{-/-}$ LSChigh cells could form adenocarcinoma (ref. 26; Fig. 6A, right) but without detectable metastasis. However, in the recipients of $C;Pten^{-/-};K-ras^{L/W}$ LSChigh cells and EpCAM\textsuperscript{low}/CD24\textsuperscript{low} mesenchymal cells, we observed similar EMT and metastatic phenotypes (Fig. 6B). PCR genotyping of resected metastatic lesions validated the presence of $C;Pten^{-/-};K-ras^{G12D/W}$ cancer cells (Supplementary Fig. S7C). Therefore, both LSChigh and EpCAM\textsuperscript{low}/CD24\textsuperscript{low} stem/progenitor cells isolated from $C;Pten^{-/-};K-ras^{L/W}$ mutant mice have enhanced prostate capacity to reconstitute EMT and drive distant metastasis compared with stem/progenitor cells with either PI3K activation or RAS/MAPK activation alone.

**Pharmacologic targeting of RAS/MAPK signaling inhibits metastatic disease initiated from stem/progenitor cells**

Because transplantation of stem/progenitor cells isolated from $C;Pten^{-/-};K-ras^{L/W}$ mutants yielded metastatic disease with reliable kinetics, we investigated whether targeting of the PI3K/AKT and RAS/MAPK signaling pathways could inhibit such a phenotype. To noninvasively monitor metastasis in vivo, we crossed the Rosa26-Luc reporter line onto the compound mutant mice so both primary and metastatic lesions can be easily monitored via bioluminescence imaging (BLI; Fig. 7A and data not shown). We first tested the ability of mTOR inhibitor rapamycin (4 mg/kg/d, intraperitoneally) and MEK inhibitor PD325901 (5 mg/kg/d, per os) to effectively inhibit the PI3K and RAS pathways in vivo, using $C;Pten^{-/-};K-ras^{L/W}$ mutant mice. As shown in Fig. 7B, left, these small-molecule inhibitors could hit their respective pathways in vivo, indicated by the reduction of their downstream surrogate markers p-S6 and p-MAPK staining. Coinciding with efficient reduction of phospho-targets, we observed marked reduction of Ki67\textsuperscript{+} cells.
in C\(^+\);Pten\(^{L/L}\);K-ras\(^{L/W}\) mutants treated with rapamycin and PD325901.

**Figure 6.** Transplantation of C\(^+\);Pten\(^{L/L}\);K-ras\(^{L/W}\) stem/progenitor cells are sufficient to initiate EMT and metastasis. A, orthotopic transplantation of C\(^+\);Pten\(^{L/L}\);K-ras\(^{L/W}\) spheres cells (left) and C\(^+\);Pten\(^{L/L}\) LSChigh cells (right) to NOD:SCID/IL2ry-null recipients and resulting pathology (left). B, transplantation of LSChigh, LSC\(^{LO}\), and mesenchymal cells isolated from primary C\(^+\);Pten\(^{L/L}\);K-ras\(^{L/W}\) mutant cells and resulting pathology observed in NOD:SCID;IL2ry-null recipients. Low magnification bar, 500 μm; high magnification bar, 100 μm.

Discussion

The study of molecular mechanisms underlying late-stage metastatic prostate cancer has been challenging partly as a result of the paucity of prostate cancer models that recapitulate the multistep process of the metastasis. While alterations in the
Figure 7. Pharmacologic targeting of RAS/MAPK signaling inhibits metastatic disease initiated from C\(^{+}\);Pten\(^{-}\),K-ras\(^{L/W}\) mutant stem/progenitor cells. A, isolation of prostate sphere cells from C\(^{+}\);Pten\(^{-}\),K-ras\(^{L/W}\),LSL-Luc mutants and orthotopic injection to NOD;SCID;IL2r\(^{-}\)-null mice. Recipients were then treated with placebo, rapamycin (R) + PD325901 (PD), or PD325901 alone. B, effect of rapamycin/PD325901 treatment on p-MAPK and p-S6 levels (left), cell proliferation (Ki67\(^{+}\) index; middle), and mesenchymal content (right). HE, hematoxylin and eosin; WT, wild-type. **, \(P < 0.005\). C, effect of rapamycin/PD325901 or PD325901 on thoracic region BLI (left, middle) and metastatic lung lesion content (right). **, \(P < 0.005\). D, model showing that Pten-null LSC\(^{high}\) cells can initiate prostate cancer and with RAS/MAPK activation lead to EMT, metastatic disease, and formation of macrometastatic lesions. MEKI, MEK inhibitor; MET, mesenchymal-to-epithelial transition. PI3Ki, PI3K inhibitor.
PTEN/P38K/AKT signaling axis occur frequently in human disease, such pathway alterations are not sufficient to manifest a significant metastatic phenotype in preclinical animal models (35-38). In this study, we identified significant enhancement of RAS signaling in both recurrent primary tumors and bone metastasis. In consideration of these findings, we evaluated the possibility that RAS/MAPK activation could serve as a critical, additional hit to alteration of PTEN/P38K/AKT signaling in promoting prostate cancer progression and metastasis (Fig. 7D). Through coordinate Pten deletion and K-rasG12D/W activation, we observed markedly enhanced tumor progression compared with Pten deletion alone. Striking features of C+;Pten+/-;K-rasL/W and C+;PtenL/W;K-rasL/W mutants included the presence of EMT and significant metastatic burden. Importantly, our study also showed that both LSChigh epithelial and EpCAMlow/C24low mesenchymal stem/progenitor cells have sphere-forming activity in vitro and could reconstitute both local invasive and distance metastatic disease in vivo.

EMT has been postulated to play a critical role in the process of metastasis (39-41). Expression of EMT markers is correlated with human prostate cancer progression as exemplified by the enhanced levels of the Twist (36) and N-cadherin (19) in late-stage primary and metastatic diseases. Moreover, monoclonal antibody targeting of N-cadherin significantly delays progression in prostate cancer xenograft models (19). However, as few preclinical prostate cancer models progress from invasive carcinoma to EMT and metastasis, the functional significance and the pathways involved with EMT have been difficult to study. Using the Ptenp53 prostate cancer model (6), a recent study derived lineage-specific cell lines which could metastasize upon orthotopic injection into immunocompromised hosts (7). However, the interpretation of these findings is confounded by the fact that primary Ptenp53 mutant tumors rarely show extensive metastasis and that derived cell lines may undergo in vitro adaptation or acquire additional mutations. Therefore, the new Pten-nullK-ras activated model provides a unique opportunity for studying the significant impact of EMT in prostate cancer progression in vivo and the pathway that regulates the EMT biology in the context of PTEN loss. Because both K-ras and B-ras alterations occur in primary and metastatic prostate cancer (2), it will be important to model these alterations and determine whether these genetic changes have distinct or overlapping roles in prostate cancer development.

Cells with qualities of stemness and invasiveness have also been postulated to confer greater therapeutic resistance, particularly, in recurrent disease (42). If true, then such a hypothesis would explain the relatively poor response that therapies have toward metastatic cancers in comparison with differentiated primary tumors. Recent studies using breast cancer cell lines treated with paclitaxel or 5-fluorouracil, showed a 5-fold increase in CD44+/CD24low cells (43) whereas primary breast cancer samples isolated from chemotherapy-treated patients showed a 7-fold increase in the same cell population (44). That the CD44+/CD24low cells share a stem cell signature and mesenchymal characteristics suggests that EMT may be involved in the formation of cancer stem cells and therapeutic resistance. In prostate cancer, such studies are far fewer in number; however, the CD44+CD24low subpopulation isolated from prostate cancer cell lines has been attributed to both stemness and invasiveness mediated by EMT (45) and correlated with poor clinical outcome in patients with prostate cancer (46). In our study, we have identified significant expansion of EpCAMlow/CD24low mesenchymal cells that have sphere-forming activity and can lead to the regeneration of primary and metastatic lesions upon orthotopic transplantation. It will be interesting to test whether this is the population responsible for therapeutic resistance.

Because our model is based on the coordinate loss of Pten and Ras activation, we tested the effectiveness of combined mTOR and MEK inhibition on stem/progenitor cell–mediated transplantations. We observed near complete inhibition of metastatic lung lesions in treatment cohorts. Previous studies have shown that combined pharmacologic targeting of mTOR and MEK may lead to reduced primary tumor progression (31). Thus, to further test that RAS/MAPK signaling serves as a critical step in the metastatic process, we also treated animals with MEK inhibitor alone. Remarkably, using only PD259901, we also observed near-complete abolishment of metastasis, possibly as a result of impeding Ras-dependent EMT (Fig. 7D). Collectively, our observations indicate that in Pten-nullRas activated prostate cancer, the RAS/MAPK pathway plays a significant role in metastasis.

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

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