Tracking and Functional Characterization of Epithelial–Mesenchymal Transition and Mesenchymal Tumor Cells during Prostate Cancer Metastasis

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Abstract

The epithelial–mesenchymal transition (EMT) has been postulated as a mechanism by which cancer cells acquire the invasive and stem-like traits necessary for distant metastasis. However, direct in vivo evidence for the role of EMT in the formation of cancer stem–like cells (CSC) and the metastatic cascade remains lacking. Here we report the first isolation and characterization of mesenchymal-like and EMT tumor cells, which harbor both epithelial and mesenchymal characteristics, in an autochthonous murine model of prostate cancer. By crossing the established Pb-Cre+/−;Pten+/−,KrasG12D+/− prostate cancer model with a vimentin-GFP reporter strain, generating CPKV mice, we were able to isolate epithelial, EMT, and mesenchymal-like cancer cells based on expression of vimentin and EpCAM. CPKV mice (but not mice with Pten deletion alone) exhibited expansion of cells with EMT (EpCAM+/Vim-GFP+) and mesenchymal-like (EpCAM−/Vim-GFP−) characteristics at the primary tumor site and in circulation. These EMT and mesenchymal-like tumor cells displayed enhanced stemness and invasive character compared with epithelial tumor cells. Moreover, they displayed an enriched tumor-initiating capacity and could regenerate epithelial glandular structures in vivo, indicative of epithelia–mesenchyme plasticity. Interestingly, while mesenchymal-like tumor cells could persist in circulation and survive in the lung following intravenous injection, only epithelial and EMT tumor cells could form macrometastases. Our work extends the evidence that mesenchymal and epithelial states in cancer cells contribute differentially to their capacities for tumor initiation and metastatic seeding, respectively, and that EMT tumor cells exist with plasticity that can contribute to multiple stages of the metastatic cascade. Cancer Res; 75(13): 1–11. ©2015 AACR.

Introduction

Prostate cancer is the most commonly diagnosed male malignancy and the second leading cause of cancer-related death in Western men (1). Although localized prostate cancer is treatable, metastatic, late-stage castration-resistant prostate cancer (mCRPC) is currently incurable and represents the major cause of prostate cancer–related death (2). Recent studies have focused on the processes and pathway alterations that cause prostate tumor cells to disseminate and metastasize. We and others have shown that activation of the PI3K/AKT and RAS/MAPK pathways is associated with metastatic prostate cancer, and that activation of both pathways is sufficient to induce distant metastasis and an epithelial–mesenchymal transition (EMT) at the primary tumor site in genetically engineered mouse models (3–5). The acquisition of an EMT phenotype within localized cancer has been demonstrated to be sufficient to trigger lethal metastatic disease (4, 6, 7) and promote CRPC (8–10) in multiple model systems.

EMT, in the context of cancer, allows epithelial cancer cells to acquire migratory and invasive characteristics, as well as overcome senescence, apoptosis, and anoikis, properties that are essential for tumor cell dissemination and distant metastasis (11). Moreover, recent studies have also implicated EMT in the acquisition of stem-like qualities (12–14) and drug resistance properties (15). However, evidence for the role of EMT in cancer stem cell formation and metastasis is mostly based on either in vitro manipulation of cultured cell lines to induce EMT or the expression of EMT signature markers in human cancer samples (16). Therefore, a direct role for EMT in prostate tumor progression, dissemination of circulating tumor cells (CTC) into the blood stream, and seeding of metastases at distant sites remains unclear due to the lack of in vivo models that recapitulate the metastatic process.

We previously reported that deletion of the Pten tumor suppressor gene and conditional activation of the KrasG12D oncogene in the murine prostate epithelium (Pb-Cre+/−;Pten+/−,KrasG12D+/−) leads to 100% penetrable macrometastasis to the lungs and liver, and an EMT phenotype at the primary tumor site (4). Although the Pb-Cre+/−;Pten+/−,KrasG12D+/− (CPK) prostate cancer mouse...
model recapitulates late-stage, metastatic human prostate cancer and associates EMT with prostate cancer metastasis, whether tumor cells that have undergone an EMT contribute directly to tumor progression, dissemination, and distant macrometastasis is yet to be established. In this study, we develop an in vivo system that allows tracking of the dynamic EMT program and isolation of cells from the CPK prostate cancer model that have either completed (mesenchymal-like) or are transitioning through an EMT for characterization and functional testing. Our in vivo analysis suggests that mesenchymal and epithelial states contribute to different stages of prostate cancer disease, and that EMT tumor cells, which have the plasticity to readily transition between epithelial and mesenchymal lineages, are able to contribute to multiple stages of the metastatic cascade.

Materials and Methods

Mouse strains

Vim-GFP reporter mice were purchased from GENSAT (16). After crossing Vim-GFP mice with the Cre+/−;Pten129;KrasG12D+ model (4), Pb-Cre+/−;Pten129;KrasG12D+Vim-GFP male mice were crossed with Pb-Cre+Pten129;KrasG12D+/− female mice to generate the Cre−;Pten129;KrasG12D+;Vim-GFP (V), Cre+/−;Pten129;KrasG12D+;Vim-GFP (CPV), and Cre+/−;Pten129;KrasG12D+;Vim-GFP (CPKV) mouse models. These strains have been maintained on a mixed strain background. All studies with animals were performed under the regulation of the division of Laboratory Animal Medicine at the University of California at Los Angeles (Los Angeles, CA).

Histology and immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissues. Antigen retrieval was performed by boiling sections in 10 mmol/L citrate buffer (pH 6) for 30 minutes. The following primary antibodies were used: vimentin (Cell Signaling Technology; 5751), GFP (Cell Signaling Technology; 2955), E-cadherin (BD Biosciences; 610181), P-S6 (Cell Signaling Technology; 5741), GFP (Cell Signaling Technology; 2955), E-cadherin (BD Biosciences; 610181), P-S6 (Cell Signaling Technology; 5741), and pan-Cytokeratin (Sigma; C1801).

Matrigel invasion assay

Transwell inserts (8 μm; BD Biosciences) were coated with Matrigel (300 μg/mL; BD Biosciences) and placed into 24-well culture plates. A total of 5 × 10⁴ sorted cells per population were resuspended in serum-free media in the top chamber, while full serum media (DMEM with 10% FBS) was used in the bottom chamber. After four hours later, invaded cells were fixed with methanol, stained with 0.2% crystal violet, and counted using a light microscope at ×10 magnification.

Matrigel sphere assay

The Matrigel sphere assay was carried out as previously described (17). A total of 5 × 10³ sorted cells from each cell population were plated in triplicate.

BrdUrd pulse labeling

5-Bromo-2'-Deoxyuridine (BrdUrd) pulse labeling was carried out as previously described (18). A total of 2 × 10⁶ FACS sorted cells per population were cytospun onto coated cytoslides (Thermo Scientific) at 500 rpm for 5 minutes using Cytopsin4 (Thermo Scientific). Cells were fixed with 4% paraformaldehyde for 15 minutes and stained with a BrdUrd (BD Biosciences; 563445) primary antibody. Ten fields per slide were counted at ×20 magnification.

Subcutaneous tumor regeneration assay

Prostate lobes/regions from CPKV mice were separated as described (Fig. 3B), serrated, mixed with Matrigel, and transplanted subcutaneously into NOD/SCID/IL2R−null (NSG) mice. Once tumors reached 2 cm in size, mice were euthanized, and tumors were again serrated, mixed with Matrigel, and passaged into new recipient hosts.

Orthotopic tumor regeneration assay

A total of 5 × 10⁵ sorted cells per population were mixed in 50% Matrigel/media, loaded into a 10 μL Hamilton syringe (Microliter), and 2.5 × 10⁵ cells were injected into each anterior lobe of the prostates of recipient NSG mice.

Tail vein injections

A total of 2.5 × 10⁶ or 1 × 10⁶ sorted cells from each population were reseeded in 200 μL of PBS and injected intravenously into NSG hosts. The presence of lung macrometastases was assessed by gross examination of formalin-fixed lung samples under a dissecting microscope.

Statistical analysis

GraphPad Prism software was used to calculate mean and SD. The Student t test was used to calculate the statistical significance between the two groups of data. P < 0.05 is considered significant.

Results

Tracking EMT and mesenchymal-like tumor cells in an endogenous prostate cancer model using a vimentin-GFP reporter line

To generate an in vivo tracking system to study the role of EMT in prostate cancer progression and metastasis, we crossed vimentin-GFP mice (16) with the Pb-Cre+/−;Pten129;KrasG12D+ (CPK) murine model of prostate cancer (4) we recently developed to create the Pb-Cre+/−Pten129;KrasG12D+/−;Vim-GFP model (CPKV). Vimentin-GFP reporter mice, in which GFP expression is driven from the endogenous vimentin promoter on a bacterial artificial chromosome (BAC; ref. 16), were chosen because vimentin is one of the earliest upregulated genes during the EMT process (19), and its expression is associated with high Gleason scores, disease recurrence, and bone metastasis in human prostate cancers (20, 21).

In 10- to 12-week-old CPKV prostate, GFP staining overlaps with endogenous vimentin expression, which marks EMT regions within the stromal compartment surrounding GFP-negative epithelial glandular structures (Fig. 1A). These EMT regions also contain cells that are PTEN+ and P-S6+, a surrogate marker for PTEN loss and activation of the PI3K pathway, confirming that these cells were originally derived from Pten-loxp/loxp prostate epithelial cells that underwent Cre recombination (Fig. 1A). As it is possible that endogenous stromal cells in the prostate, including CD45 leukocytes, also express P-S6+, we stained prostate sections from 4 week old CPKV mice, a time point when these mice have not yet developed invasive prostate tumors or an EMT phenotype, to identify whether vimentin−/GFP+ stromal cells in the prostate normally express P-S6+. Indeed, vimentin−/GFP−
stromal cells in these prostates were PTEN$^+$ and P-S6$^-$ (Supplementary Fig. S1A). Moreover, in WT Vim-GFP (V) prostates, all vimentin$^+/GFP^+$ stromal cells are also P-S6$^-$ (Supplementary Fig. S1B). These results verify that P-S6 can be used as a marker in our CPKV model to distinguish vimentin$^+/GFP^+/PTEN^+$ stromal cells from vimentin$^+/GFP^+/PTEN^-$ EMT cells derived from the Pten$^{-/-}$ prostate epithelium.

To isolate and characterize tumor cells with epithelial and mesenymal characteristics from the prostates of CPKV mice, a FACS-gating strategy was designed in which the epithelial cell adhesion molecule (EpCAM) was used as an epithelial marker and GFP as a mesenymal marker (Supplementary Fig. S1A). Cells from CPKV prostates were first negatively selected from CD45$^-$, CD31$^-$, and Ter119$^+$ fractions (referred to as Lin$^-$) to avoid contamination from leukocyte, endothelial, and erythrocyte populations, respectively, as these cell types are known to express vimentin (19). While age-matched WT Vim-GFP (V) (n = 8) and Pb-Cre$^{+/--;Pten^{+/--};Vim-GFP (CPV)}$ (n = 8) mutant prostates have a very rare population of cells that are EpCAM$^+/GFP^+$, which we will refer to as mesenchymal-like tumor cells, CPKV mutants (n = 13) have a significant induction of the mesenchymal-like tumor cell population by 10 weeks of age (Fig. 1B and Supplementary Fig. S1C). Interestingly, a significant population of cells that coexpress both epithelial and mesenchymal markers (EpCAM$^+/GFP^+$), hereafter referred to as EMT cells, could also be isolated from 10- to 12-week-old CPKV, and, to a small extent, CPV prostates, but not V prostates (Fig. 1B and Supplementary Fig. S1C). While there was a marginal increase in the percentage of EpCAM$^+/GFP^+$ epithelial cells within CPV and CPKV prostates compared with V prostates, this change was not significant.

Figure 1.
Tracking EMT and mesenchymal-like tumor cells in an endogenous prostate cancer model using a vimentin-GFP reporter line. A, prostates from CPKV mice (12 weeks) display EMT regions that are Vim-GFP-positive surrounding E-cadherin (E-cad) (red) and P-S6 (green) and E-cadherin (E-cad) (red) were found within epithelial glandular structures in CPKV prostates (12 weeks). E, qPCR analysis confirmed EMT signature gene expression in EMT and mesenchymal-like tumor cells isolated from CPKV prostates (10–12 weeks). F, Matrigel invasion assay revealed that EMT and mesenchymal-like tumor cells isolated from CPKV prostates (10–12 weeks) are significantly more invasive than epithelial tumor cells.

Data in B, C, E, and F are represented as mean ± SEM. Bar, 50 μm. P<0.05; **, P<0.001; ***, P<0.0001.

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(Fig. 1B and Supplementary Fig. S1A). Genomic PCR analysis confirmed that all three FACS-isolated tumor cell populations were indeed derived from epithelial cells that initially underwent Cre recombination, as they exhibit Pten deletion and Kras activation (Supplementary Fig. S1D).

To assess the association of the EMT and mesenchymal-like tumor cell populations with prostate cancer progression, we compared 10- to 12-week-old CPKV mutants \( (n = 11) \), which have begun to develop poorly differentiated EMT morphology, to late-stage 15- to 20-week-old mutants \( (n = 9) \) with sarcomatoid morphology \( (4) \). While there was a significant expansion of mesenchymal-like tumor cells during tumor progression, there was no change in the percentage of EMT tumor cells in the 15- to 20-week-old mutants (Fig. 1C), indicating that EMT tumor cells may indeed represent a transition stage. Histologically, rare EMT tumor cells that coexpressed both epithelial (E-cadherin; red) and mesenchymal markers (Vim-GFP; green) were found within epithelial acinar structures, indicating that the early steps of the EMT process do not occur exclusively along the leading edge of the tumor (Fig. 1D, yellow arrows).

Finally, we confirmed that EpCAM\(^+\)/GFP\(^+\) and EpCAM\(^-\)/GFP\(^+\) cell populations isolated from CPKV prostates indeed display EMT signature gene expression by qPCR analysis. While both EMT and mesenchymal-like tumor cells had induced expression of EMT signature genes compared with epithelial tumor cells, mesenchymal-like prostate tumor cells displayed more dramatically increased expression of the EMT transcription factors Snail and Zeb1, as well as a switch from E-cadherin (Cdh1) to N-cadherin (Cdh2) expression (Fig. 1E). Mesenchymal-like tumor cells were also significantly more invasive ex vivo compared with epithelial tumor cells, with EMT tumor cells displaying an intermediate invasive capacity (Fig. 1F). Taken together, we have developed a novel system by using a Vim-GFP reporter to faithfully track the dynamics of the EMT program and to isolate and characterize cells undergoing or having undergone an EMT in an endogenous prostate cancer model.

EMT and mesenchymal-like tumor cells have enhanced stemness properties

To explore whether EMT and mesenchymal-like tumor cells from an endogenous prostate cancer model have enhanced stemness qualities, tumor cell populations from primary CPKV prostates were isolated by FACS and grown in Matrigel cultures to assess their ability to form spheres, an assay that has been used to evaluate the potential of tumor cell populations with prostate cancer progression, we compared 10- to 12-week-old CPKV mutants \( (n = 11) \), which have begun to develop poorly differentiated EMT morphology, to late-stage 15- to 20-week-old mutants \( (n = 9) \) with sarcomatoid morphology \( (4) \). While there was a significant expansion of mesenchymal-like tumor cells during tumor progression, there was no change in the percentage of EMT tumor cells in the 15- to 20-week-old mutants (Fig. 1C), indicating that EMT tumor cells may indeed represent a transition stage. Histologically, rare EMT tumor cells that coexpressed both epithelial (E-cadherin; red) and mesenchymal markers (Vim-GFP; green) were found within epithelial acinar structures, indicating that the early steps of the EMT process do not occur exclusively along the leading edge of the tumor (Fig. 1D, yellow arrows).

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Prostate regions enriched in Vim-GFP\(^+\) cells are able to regenerate transplantable tumors in vivo

As prostate stem cells have been previously shown to reside in the proximal region of the prostate attached to the urethra \( (23) \), we next wanted to ascertain whether EMT and mesenchymal-like tumor cells were also enriched in this stem cell niche. Gross fluorescent imaging of whole CPKV prostates revealed that Vim-GFP expression was most prominent in the proximal anterior lobes, and, interestingly, in the anterior portion of the urethra itself (Fig. 3A). When distinct anatomical regions of the prostate were separated from 10-week-old CPKV mice with established tumors (Fig. 3B) and subjected to quantitative FACS analysis, the percentage of mesenchymal-like tumor cells was significantly higher in the proximal region of the anterior lobes and the anterior portion of the urethra, the regions immediately connected to the proximal region of the prostate, compared with other regions of CPKV prostates (Fig. 3C, right panel, \( n = 3 \)). Although the percentage of EMT tumor cells was slightly higher in the proximal region of anterior lobes, the percentage of EMT tumor cells did not change dramatically between the different regions of the prostate (Fig. 3C, left panel, \( n = 3 \)). These data suggest that mesenchymal-like tumor cells may indeed have properties of quiescent stem cells, which are localized to specific stem cell niches.

To test whether the regions of CPKV prostates with the highest percentage of EMT and mesenchymal-like tumor cells also have the highest tumor-initiating capacity, regions/lobes were separated from 10-week-old CPKV prostates, mixed with Matrigel, and implanted subcutaneously into NOD/SCID/IL2R\(^-\) (NSG) mice. Remarkably, the only regions that were able to form subcutaneous tumors in vivo were from the proximal region of the anterior gland and anterior portion of the urethra, the same regions with the highest percentage of EMT and mesenchymal-like tumor cells (Fig. 3D, \( n = 4 \)). Tumors from both regions were able to be serially passaged, and these passaged tumors contained all 3 prostatic cell lineages (CK5\(^+\) basal, CK8\(^+\) luminal, and Synaptophysin\(^+\) neuroendocrine cells; Supplementary Fig. S2), demonstrating that stem cells within these regions have the capacity to regenerate all prostate lineages.

Klf2 and Klf4, the self-renewal maintenance factors Bmi1, Nestin, and Lrig1, and members of the WNT and NOTCH developmental pathways, suggesting that mesenchymal-like tumor cells may also harbor stemness qualities (Fig. 2C and D). One possible explanation for their poor sphere-forming capacity may be that mesenchymal-like tumor cells exist in a quiescent state and therefore have less capacity to proliferate and form sphere structures \( (23, 24) \). To test this hypothesis, we pulse labeled CPKV mice with BrdUrd for 24 hours before FACS sorting to assess the percentage of cycling cells in each population. While the epithelial and EMT tumor cell populations had a high percentage of cells in S-phase, the mesenchymal-like tumor cell population had a much lower percentage of cycling cells (Fig. 2E). In support of this finding, our K667 immunohistochemical analysis revealed that E-cadherin–positive glandular structures are more proliferative than GFP-positive mesenchymal regions in the stroma (Fig. 2F). In summary, both EMT and mesenchymal-like tumor cells from CPKV mutants have enhanced stemness characteristics, with EMT tumor cells displaying a proliferative, stem/progenitor cell phenotype, and mesenchymal-like tumor cells exhibiting a quiescent stem cell phenotype.
EMT and mesenchymal-like tumor cells have enhanced tumor-initiating capacity and cellular plasticity in vivo

To directly assess the tumor-initiating capacity of epithelial, EMT, and mesenchymal-like tumor cells isolated from CPKV mice, 5,000 sorted cells from each population were implanted orthotopically into the anterior lobes of NSG mice and allowed to incubate up to 6 months (Fig. 4A). Tumor pathology was defined histologically by the presence of abnormal glandular structures, stromal expansion, and positive staining for P-S6 and Ki67, which should not be present in normal prostate tissue (Fig. 4C and D and Supplementary Fig. S3A). While EMT and mesenchymal-like tumor cells have a high tumor-initiating capacity in vivo, with 6 of 9 and 7 of 9 mice injected with these tumor cells forming prostate tumors, respectively, only 3 of 13 mice injected with epithelial tumor cells formed tumor pathology in vivo (Fig. 4B and Supplementary Fig. S3A). EMT and mesenchymal-like tumor cells also formed tumors as early as 9 weeks and 6 weeks posttransplantation, respectively, whereas the epithelial tumor cells did not form tumors until 24 weeks posttransplantation (Supplementary Fig. S3B). Tumorigenic areas formed from transplanted epithelial tumor cells were also much less aggressive compared to those formed by EMT and mesenchymal-like tumor cells (T; Supplementary Fig. S3A). EMT and mesenchymal-like tumor cells generated tumors with vast regions of EMT morphology that were vimentin-negative and P-S6-positive (Fig. 4C and D, bottom panels). Despite initially being injected as GFP+ cells, EMT and mesenchymal-like tumor cells were also remarkably able to regenerate tumors in mouse models of prostate intraepithelial neoplasia lesions that were vimentin-negative and P-S6-positive (Fig. 4C and D, top), indicating that these cells have the plasticity to switch between mesenchymal and epithelial states. In general, while regenerated epithelial glandular structures...
were positive for proliferation markers (Ki67), invasive EMT regions were devoid of any proliferation markers in tumors generated from EMT and MES-like tumor cells (Fig. 4C and D), mimicking the phenotype seen in primary CPKV prostates (Fig. 2F). These results demonstrate that EMT and mesenchymal-like tumor cells have enhanced tumor-initiating capacity compared with epithelial tumor cells from the same CPKV mice, and that these cells have an inherent plasticity to switch between mesenchymal and epithelial states, as they are able to form invasive tumor regions that are P-S6+, vimentin+, and Ki67hi, as well as regenerative glandular structures that are P-S6-, Ki67lo/hi, and vimentin- (Fig. 4E).

Increase in CTCs with mesenchymal-like and invasive characteristics during disease progression in CPKV mice

CTCs represent a surrogate biomarker of metastatic disease and a predictive factor of overall survival in various malignancies, including prostate cancer (25, 26). The current FDA-approved CellSearch method for CTC enumeration uses antibodies against EpCAM to isolate CTCs with epithelial characteristics (27). However, recent studies in human breast and prostate cancer patients have revealed that a significant percentage of CTCs coexpress both mesenchymal and epithelial markers or display fully mesenchymal characteristics (21, 28–30), through which they could pass undetected by the CellSearch system.

To determine whether we could use the Vim-GFP reporter to isolate and characterize CTCs with EMT and mesenchymal-like characteristics at different disease stages, we collected peripheral blood from CPKV mutants. CTCs were isolated and characterized from the Lin- population by FACS analysis in a similar manner to how the primary tumor cell populations were isolated (Supplementary Fig. S4A). As early as 6 weeks of age and well before the detection of metastatic disease, epithelial, EMT and mesenchymal-like CTCs can be detected in the blood of CPKV mice (Fig. 5A,

![Figure 3](https://example.com/figure3.png)
left, n = 7). During the intermediate (10–12 weeks) and late stages (15–20 weeks) of tumor progression, there is a significant expansion of mesenchymal-like CTCs and rare EMT CTCs but not epithelial CTCs in CPKV mice, suggesting that mesenchymal-like and EMT CTC counts correlate with progression towards metastatic disease (Fig. 5A, middle and right panels, n = 10). Supporting this finding, CPV mice, which develop micrometastases in the lymph nodes but not distant macrometastasis to the lung or liver (31), show similar epithelial CTC numbers to age-matched CPKV mice but have very few CTCs with mesenchymal-like characteristics and no CTCs with EMT traits (Fig. 5A, n = 5). These data suggest that (i) dissemination of metastatic tumor cells occurs early on during tumor initiation, (ii) mesenchymal-like and EMT CTC counts correlate with metastatic disease, and (iii) EMT tumor cells may quickly polarize to a mesenchymal or epithelial phenotype upon entering blood circulation.

As it is thought that disseminated cells with metastatic seeding potential must possess stem cell characteristics to colonize distant tissues, we looked at expression of CD44, a putative CSC marker (32), in our different CTC subpopulations. Throughout the various stages of disease progression in CPKV mice, the majority of CTCs with mesenchymal-like characteristics also expressed CD44, while only a small percentage of epithelial CTCs expressed this CSC marker (Fig. 5B, n = 10). Interestingly, 100% of rare EMT CTCs that could be detected were CD44<sup>+</sup> (Fig. 5B, n = 10).

To further assess the metastatic seeding potential of the CTC subpopulations, blood from CPKV mutants, as well as CPV and V mutants, was incubated on Vitatex culture plates, which are coated with a cell-adhesion matrix (CAM) that is used to assess the ability of tumor cells to invade collagen matrices (33, 34). Utilizing a Vitatex plate coated with a fluorescent red CAM, we are able to identify invasive CTCs (iCTCs), which represent CTCs with metastatic potential, by their ability to invade through the matrix and ingest TRITC-labeled CAM (Supplementary Fig. S4B). After 10 days in culture, blood isolated from CPKV mice (n = 10) had a significantly larger number of TRITC<sup>+</sup> iCTCs compared with blood from CPV (n = 5) or V mice (n = 3), supporting the notion that CTCs in CPKV mice contain more metastatic seeding potential (Fig. 5C). Moreover, when exploring the phenotype of these iCTCs from CPKV mice by FACS analysis, the majority of iCTCs had mesenchymal-like characteristics (Fig. 5D). The Vim-GFP reporter can therefore also be utilized for the isolation and characterization of CTC populations during endogenous prostate tumor cell dissemination and metastatic spreading. Our analysis
reveals that CPKV mice have a significant increase in EMT and mesenchymal-like CTCs, but not epithelial CTCs, during disease progression.

Epithelial tumor cells have enhanced metastatic seeding potential

The observation that metastatic lesions in humans often display an epithelial morphology suggests that tumor cells that have disseminated through an EMT may revert to an epithelial phenotype through a MET to form macrometastases (35). To test whether an MET may be required for macroscopic metastasis in CPKV mice, we compared epithelial and mesenchymal marker expression in both micrometastases, which remain small, dormant lesions, and actively proliferating macrometastases in the lungs. While micrometastases express high levels of vimentin and low levels of pan-cytokeratin (CK) and Ki67, macrometastases express low levels of vimentin and high levels of CK and Ki67 (Fig. 6A). These data suggest that a reversion to an epithelial phenotype, marked by high levels of CK, may be required for dormant micrometastases to proliferate to form macrometastases.

We next wanted to directly test the metastatic seeding capacity of epithelial, EMT and mesenchymal-like tumor cell populations by injecting these primary tumor cell populations isolated from CPKV mice intravenously into NSG mice. Remarkably, while mesenchymal-like tumor cells were unable to form macrometastases up to 16 weeks posttransplantation, epithelial tumor cells readily formed macrometastases when either 25,000 (2 of 5) or 100,000 (6 of 6) tumor cells were injected (Fig. 6B and C). EMT tumor cells were also able to form metastases (1 of 5), albeit at a lower efficiency compared with epithelial tumor cells (Fig. 6B and C). Upon histologic examination of the lungs posttransplantation, the macrometastases formed by both epithelial and EMT tumor cells were devoid of GFP+ cells and expressed high levels of CK (Supplementary Fig. S5), similar to lung macrometastases found in CPKV mice (Fig. 6A), confirming that macrometastatic spread requires reversion to an epithelial phenotype. While mesenchymal-like tumor cells did not form macrometastases or even small micrometastases in the lungs, solitary GFP+ cells were found in a quiescent, non-proliferative state (Ki67−) throughout the lungs (Supplementary Fig. S5). Interestingly, while few tumor cells remained in the circulation of mice transplanted with epithelial and EMT tumor cells 16 weeks after intravenous injection, those transplanted with mesenchymal-like tumor cells had a significantly high number of CTCs with mesenchymal-like characteristics, suggesting that mesenchymal-like cells can persist and survive in the blood stream for long periods of time (Fig. 6D, n = 4). These findings propose that while mesenchymal tumor cells are able to survive in the blood, extravasate, and persist as solitary, dormant cells in the lungs over the course of 16 weeks, they are unable to proliferate to generate frank metastases. EMT tumor cells, on the other hand, reside in a transitionary state, and have the capacity to transition to an epithelial state to proliferate and form macrometastases. Overall, while both EMT and mesenchymal-like prostate tumor cells have enhanced stemness characteristics and tumor-initiating capacity compared with epithelial tumor cells, only EMT tumor cells have the capacity to revert to an epithelial state and proliferate in the lungs to form macrometastases (Fig. 6E).

Discussion

As EMT is a plastic and dynamic process, the study of the EMT process through the in vitro manipulation of established cell lines, which often polarizes cells into a fixed mesenchymal state, may overlook much of the biology involved within the transition. Here, we demonstrate for the first time the isolation and characterization of both mesenchymal-like tumor cells that have fully completed an EMT, as well as EMT tumor cells that are in a transitory state between epithelial and mesenchymal programs from an endogeneous murine cancer model. While previous studies have suggested that partial but not complete passage of cells into a mesenchymal state is associated with stemness and tumorigenicity (36, 37, 38), our study reveals that both EMT and mesenchymal-like tumors cells harbor stemness characteristics
and tumor-initiating capacity. A striking distinction between these populations is their proliferative capacity, with mesenchymal-like tumor cells exhibiting characteristics of quiescent stem cells, and EMT tumor cells exhibiting characteristics of proliferating progenitor cells. Given that mesenchymal-like tumor cells localize to the stem cell niche in the proximal region of the prostate, it is likely that they are maintained in a quiescent state by factors in the surrounding microenvironment (24).

While both EMT and mesenchymal-like tumor cells demonstrate the plasticity to initiate proliferative epithelial tumor structures in the prostate microenvironment, only EMT cells are able to quickly revert to an epithelial phenotype to form macrometastatic colonies in the lungs. This suggests that mesenchymal-like tumor cells may exist in a more fixed state compared with EMT tumor cells, and may require additional stimuli, possibly mediated through paracrine factors secreted from the surrounding microenvironment, to acquire an epithelial phenotype and proliferate to form distant metastases. The modeling of dissemination in immunodeficient mice in our study, as well as by others (36, 39), presents a number of caveats that may affect the rate of successful metastatic colonization. First, NSG mice lack mature T cells, B cells, and NK cells, and have documented defects in macrophage activation. This is problematic, as the outgrowth of macrometastases has been shown to rely on the successful recruitment of myeloid and other inflammatory cell types to the metastatic site (40). Second, primary tumors release systemic factors that can educate distant sites in preparation for metastasizing cancer cells and mobilize bone marrow-derived cells to these sites to orchestrate what is termed the “premetastatic niche” (41–43). The lack of both a primary tumor and various immune cell subtypes in our

**Figure 6.** Epithelial tumor cells have enhanced metastatic seeding potential. A, IHC analysis of epithelial (Pan-CK), mesenchymal (Vim), and proliferation (Ki67) markers in micrometastases (micromets) and macrometastases (macromets) in primary CPKV lungs (18 weeks). Low magnification bar, 500 μm; high magnification bar, 50 μm. B, percent of NSG mice with macrometastatic lesions in the lungs 16 weeks after intravenous transplantation of either 25,000 or 100,000 epithelial, EMT or mesenchymal-like tumor cells from CPKV prostates (10–12 weeks). C, whole mount images of lungs isolated from NSG mice transplanted with 25,000 epithelial, EMT or mesenchymal-like tumor cells from CPKV prostates (10–12 weeks). Circle, macrometastases. Bar, 4 mm. D, NSG mice transplanted intravenously with 25,000 mesenchymal-like tumor cells from CPKV prostates (10–12 weeks) contained a significantly higher number of CTCs persisting in the bloodstream compared to mice transplanted with either epithelial or EMT tumor cells 16 weeks posttransplantation. E, table summarizing the characteristics of epithelial, EMT and mesenchymal (MES)-like tumor cells isolated from CPKV prostates. Data in D, mean ± SEM. *, P < 0.05.

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<td>+</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>Metastatic seeding capacity</td>
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</table>
dissemination model may indeed affect the metastatic niche required for colonization. Future studies exploring the contribution of distinct tumor cell populations to metastatic colonization will need to be carried out using lineage-tracing techniques in endogenous mouse models or through transplantation of tumor cells into immunocompetent syngeneic mice to fully recapitulate the role of the tumor microenvironment in metastatic colonization. Finally, as human prostate cancer metastasizes most frequently to the bone, it is plausible that the mesenchymal state, as opposed to the epithelial state, may be favored for metastatic colonization in the bone microenvironment. Indeed, a number of studies have demonstrated that human prostate cancer bone metastases have downregulation of E-cadherin and increased mesenchymal marker expression (44–47).

Given that mesenchymal-like CTCs increase in number significantly during disease progression in CPKV mice and harbor stemness properties, it is likely that mesenchymal-like tumor cells can settle in the distant organ sites and eventually form macrometastases, albeit over long latency periods. It has been well documented in the clinic that disseminated tumor cells (DTC) can persist in a quiescent state at metastatic sites for decades after surgical removal of the primary tumor and eventually give rise to macrometastases (48, 49). It is possible that disseminated mesenchymal-like tumor cells in our CPK mouse model define the clinical phenomenon of metastatic dormancy, and that the observation window of our metastasis assay (16 weeks) was not long enough to observe reactivation of these dormant, solitary DTCs to produce growing macrometastases. Moreover, while epithelial tumor cells can readily form metastases in the lungs after entering the bloodstream, it is unlikely that they can actively disseminate from the primary tumor in the endogenous setting. However, we cannot rule out the possibility that epithelial and mesenchymal-like tumor cells dynamically interact at different stages of the invasion–metastasis cascade to produce macrometastatic disease (36, 39). On the other hand, EMT tumor cells, by harboring the plasticity to readily transition between epithelial and mesenchymal states, have the capacity to complete the entirety of the invasion–metastasis cascade on their own. A preliminary transcriptional profiling study of each cell population revealed significant alterations in a number of key epigenetic regulators in the EMT and mesenchymal-like tumor cell populations, including HMG2A, which we found to be (i) significantly upregulated in human mCRPC, (ii) required for epithelial–mesenchymal plasticity, and (iii) successfully targeted by the histone deacetylase inhibitor (HDACi) LBH589 for therapeutic benefit in vivo (data in preparation). While more detailed studies are warranted and still ongoing, these findings provide strong support for the use of therapeutic agents that target prostate tumor cell plasticity, as opposed to therapies that specifically target the epithelial or mesenchymal state. Although therapeutic targeting of mesenchymal-like tumor cells and the mesenchymal state could be useful in preventing tumor progression and dissemination, such therapeutic strategies could be counterproductive in patients that have DTCs or dormant micrometastases, as promoting reversion to an epithelial state could in turn promote metastatic growth. Further understanding of the mechanisms regulating epithelial–mesenchymal plasticity will help to unveil novel therapies that can be used to target tumor cell plasticity and hence inhibit integral steps of the metastatic cascade that ultimately leads to prostate cancer mortality.

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

Authors’ Contributions
Conception and design: M. Ruscetti, H. Wu
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Ruscetti, B. Quach, E.L. Dadashian, D.J. Mulholland
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Ruscetti, B. Quach, E.L. Dadashian, H. Wu
Writing, review, and/or revision of the manuscript: M. Ruscetti, B. Quach, D.J. Mulholland, H. Wu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Ruscetti
Study supervision: M. Ruscetti, H. Wu

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