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TITLE: Identification and Targeting of Candidate Pre-Existing Lurker Cells that Give Rise to Castration-Resistant Prostate Cancer

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Identification and Targeting of Candidate Pre-Existing Lurker Cells that Give Rise to Castration-Resistant Prostate Cancer

The purpose of this project is to evaluate the role of a rare subset of progenitor-like luminal cancer cells that may have special properties enabling them to resist standard therapies and mediate tumor recurrence. Our most significant accomplishment to date is identifying activated inflammatory signaling in these luminal progenitor cells. Blockade of this pathway depletes growth and survival of luminal progenitor cells by reducing expression of a number of important proliferative and anti-apoptotic factors including BCL2 and eIF4E. Over-expression of eIF4E, previously found to regulate luminal progenitor-like prostate cancer cells, can partially compensate for inhibition of NFkB signaling, suggesting that eIF4E and NFkB are both regulators of this rare cell population and may act as part of the same signaling pathway. Further understanding of these progenitor cells, the pathways they use to control their growth and survival, and the markers they uniquely express will allow us to identify and target these cells in order to predict, prevent or treat advanced castration-resistant prostate cancer.
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1. INTRODUCTION
Castration-resistant prostate cancer (CRPC) is a lethal disease. We lack standard biomarkers to predict hormonal sensitivity of tumors, and we need new targets for therapy to prevent or treat CRPC. The adaptation theory predicts that pre-existing androgen-independent prostate cancer cells (termed lurker cells) contribute to CRPC, as they are not targeted by androgen-deprivation therapy and may be capable of regenerating the tumor. Therefore, targeting these predicted lurker cells in combination with androgen-deprivation therapy would likely prevent or delay the onset of CRPC. The goals of this proposal are to provide functional evidence for intermediate luminal progenitor cells as the pre-existing “lurker” cells in primary prostate tumors, to evaluate potential therapeutic targets in intermediate luminal progenitor cells, and to define candidate biomarkers in intermediate luminal progenitor cells that can predict prognosis and response to hormonal therapy.

2. KEYWORDS
Prostate, epithelial, Androgen-deprivation therapy, Castration-resistant prostate cancer, luminal progenitor, intermediate cell, lurker cell

3. ACCOMPLISHMENTS
What were the major goals of the project?

Specific Aim 1. Interrogation of human I/LP cells as candidate lurker cells. Time frame: months 1-24. This Aim is 40-50% complete as tumors have been initiated in Task 1 but further analysis will be collected in year 2 and a new approach will be added to Tasks 2 and 3 in year 2.

Task 1. Test the in vivo effects of castration on human luminal cancer subsets isolated from primary regenerated prostate tumors.

Task 2. Test the in vivo growth capacity of human luminal cancer subsets isolated from primary clinical prostate tumors.

Task 3. Test the in vivo effects of castration on human luminal cancer subsets isolated from primary clinical prostate tumors.

Specific Aim 2. Proof of principle therapeutic targeting of I/LP cells to prevent CRPC. Time frame: months 1-36. This aim is 25% complete as data has been emerging from Tasks 4 and 5.

Task 4. Determine if I/LP cancer cells from primary clinical human prostate tumors express genes associated with CRPC.


Task 6. Target I/LP cells in vivo.

Specific Aim 3. Diagnostic potential of I/LP cells as lurker cells to predict CRPC development. Time frame: months 1-36. This aim is 33% complete as Task 7 is accomplished while 8 and 9 will be performed in future years.

Task 7. Determine whether I/LP cell transcriptional signature can predict patient prognosis.

Task 8. Testing of candidate I/LP biomarkers on prostate cancer tissue.

Task 9. Determine if I/LP biomarkers can predict hormone sensitivity.
What was accomplished under these goals?

Specific Aim 1. Interrogation of human I/LP cells as candidate lurker cells.
1) major activities: Experimental tumors were initiated using oncogenes Myc and myrAKT and propagating/fractionation studies have been initiated. In addition, new oncogene-driven models of human prostate cancer are being developed with different oncogene combinations that will allow us to determine if a range of human prostate tumors demonstrate consistent or distinct results.

2) specific objectives: (1) Test the in vivo effects of castration on human luminal cancer subsets isolated from primary regenerated prostate tumors, (2) Test the in vivo growth capacity of human luminal cancer subsets isolated from primary clinical prostate tumors, (3) Test the in vivo effects of castration on human luminal cancer subsets isolated from primary clinical prostate tumors.

3) significant results or key outcomes: Early results indicate that the I/LP fraction from experimental tumors can generate new tumors more readily than the remaining cancer cells. More data will be collected as these experiments are ongoing. We are incorporating a new tissue-slice approach into Aim 1 in order to measure the number and percentage of I/LP cells in primary human prostate tumors before and after castration (see below).

4) other achievements: Methodologies for Tasks 2/3 have been updated following our initial attempts. Low numbers of sorted cancer cells have difficulty surviving after transplantation. Other groups have also failed to consistently demonstrate tumor re-growth from dissociated cancer cells (Domingo-Domenech et al, Cancer Cell 2012: tumors formed in only 4 out of 30 samples attempted). However, we have identified a methodology that will enable us to answer these questions of whether I/LP cells preferentially survive androgen-deprivation. We are utilizing a tissue-slice approach reported by Donna Peehl’s group whereby pieces of intact human prostate cancer tissue can be implanted under the renal capsule of immunodeficient intact or castrated mice (Zhao et al, Am J Pathol 2010). Analysis of tissues from intact/castrated mice will enable the proposed measurements and determination if I/LP cells are enriched in castration-resistant tissue.

Specific Aim 2. Proof of principle therapeutic targeting of I/LP cells to prevent CRPC.
1) major activities: I/LP and remaining luminal cells from primary patient tissue have been analyzed at the RNA and protein levels.
Early results indicate elevated NFkB signaling in I/LP cells leading us to inhibit this pathway and demonstrate a functional role for NFkB signaling and eIF4E-driven protein translation in I/LP cell growth and survival.

2) specific objectives: (1) Determine if I/LP cancer cells from primary clinical human prostate tumors express genes associated with CRPC. (2) Determine regulators of I/LP clonogenic activity in vitro. (3) Target I/LP cells in vivo.

3) significant results or key outcomes: Preliminary analysis identifies NFkB activation increased in I/LP cells compared to luminal cells (Figure 1). These findings suggest that I/LP cells may rely on NFkB signaling for survival and growth.

As reported in our recent PNAS paper, human prostate AR-low luminal progenitor cancer cells driven by oncogenes Myc and myrAKT are dependent on protein translation factor eIF4E and inhibition of this pathway prevents cell survival and clonogenicity (PNAS study attached, see Figure 6).
Based on initial findings in Task 4, we have tested in vitro growth of I/LP cells in the presence or absence of an NFkB inhibitor called ACHP. Clonogenic assays reveal a dramatic inhibition of growth (Figure 2).

We found that NFkB inhibition in I/LP cells is sufficient to reduce NFkB activation (p65 phosphorylation) and suppress expression of NFkB targets TNFR1 and BCL2 by western blot (Figure 3).

Mechanistically, we have found that NFkB inhibition causes reduction in elf4E, which we know is an important growth regulator and has been shown to be a transcriptional target of NFkB (Fig 3).

Growth defects caused by inhibition of NFkB can be partially rescued by over-expression of elf4E (Figure 4). These results suggest that we have identified two regulators of I/LP growth, NFkB signaling and elf4E, which may be part of the same growth-promoting pathway.

4) other achievements: Nothing to Report

Specific Aim 3. Diagnostic potential of I/LP cells as lurker cells to predict CRPC development.

1) major activities: The gene signature of I/LP and non-progenitor luminal cells was used to stratify patient outcome in a Watchful Waiting cohort to determine if I/LP transcriptional signature has predictive power.

2) specific objectives: (1) Determine whether I/LP cell transcriptional signature can predict patient prognosis. (2) Testing of candidate I/LP biomarkers on prostate cancer tissue. (3) Determine if I/LP biomarkers can predict hormone sensitivity.

3) significant results or key outcomes: 281 men with prostate cancer were followed for 30 years in a Swedish watchful waiting cohort (Sboner et al, BMC Medical Genomics 2010). Preliminary data from this cohort suggests that the I/LP signature is associated with worse overall survival compared to the non-progenitor luminal cell signature (Figure 5).

4) other achievements: Nothing to Report
What opportunities for training and professional development has the project provided?
The Principal Investigator was invited to participate and present data at the Prostate Cancer Foundation’s Coffey-Holden Prostate Cancer Academy in June 2014 focused on “beyond immune therapy…”

How were the results disseminated to communities of interest?
Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?
Nothing significant to report as experiments are still ongoing and results need to be verified in larger cohorts. Our findings do point to a role for inflammatory signaling in progenitor-like cancer cells, which may represent a new therapeutic target or at least provide additional evidence to support this pathway as a target in advanced cancer.

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
Nothing to Report

5. CHANGES/PROBLEMS

Changes in approach and reasons for change
As described above and previously reported (Domingo-Domenech et al, Cancer Cell 2012), low numbers of sorted primary cancer cells have difficulty surviving after transplantation. However, we have identified a methodology that will enable us to answer these questions of whether I/LP cells preferentially survive androgen-deprivation. We are utilizing a tissue-slice approach reported by Donna Peehl’s group whereby pieces of intact human prostate cancer tissue can be implanted under the renal capsule of immunodeficient intact or castrated mice (Zhao et al, Am J Pathol 2010). Analysis of tissues from intact/castrated mice will enable the proposed measurements and determination if I/LP cells are enriched in castration-resistant tissue. This approach will complement our initial methodology described in Tasks 2/3.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report

Changes that had a significant impact on expenditures
Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report
Significant changes in use or care of human subjects
Nothing to Report

Significant changes in use or care of vertebrate animals.
Nothing to Report

Significant changes in use of biohazards and/or select agents
Nothing to Report

6. PRODUCTS

Publications, conference papers, and presentations
Work describing the role of luminal progenitor-like cancer cells in prostate cancer propagation and a requirement for eIF4E signaling in their cell survival was reported in a 2013 PNAS article. Newer data on the pathways expressed in these cells that was collected during the first year while receiving funds from this award was presented at a conference in San Diego, CA.

Journal publications.
Stoyanova T, Cooper AR, Drake JM, Liu X, Armstrong AJ, Pienta KJ, Zhang H, Kohn DB, Huang J, Witte ON, Goldstein AS. Prostate cancer originating in basal cells progresses to adenocarcinoma propagated by luminal-like cells. PNAS USA (2013), 110:20111-20116. Federal support was not acknowledged because the study was completed prior to the initiation of DOD funds.

Liu X, Goldstein AS. Inflammation promotes prostate differentiation. PNAS USA (2014), 111:1666-7. Funding was not acknowledged for this preview/summary article.

Books or other non-periodical, one-time publications.
Nothing to Report

Other publications, conference papers, and presentations.
The Principal Investigator Andrew Goldstein presented his work, “Epithelial progenitor cells and inflammation” during a plenary session (Game Changing Research from PCF High-Achieving Young Investigators) at the Prostate Cancer Foundation 20th annual meeting in Washington, DC in October, 2013. The PI also presented his work, “Prostate epithelial heterogeneity and immune cell interactions” at the Prostate Cancer Foundation’s Coffey-Holden Prostate Cancer Academy held in San Diego, CA in June, 2014.

Website(s) or other Internet site(s)
Nothing to Report

Technologies or techniques
Nothing to Report

Inventions, patent applications, and/or licenses
Nothing to Report

Other Products
Nothing to Report
7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name: Andrew Goldstein
Project Role: PI
Nearest Person Month Worked: 3
Contribution to Project: Initiation of tumors in Aim 1, analysis of I/LP cells in Aim 2

Name: Xian Liu
Project Role: Technician/Staff Research Associate
Nearest Person Month Worked: 3
Contribution to Project: Initiation of tumors in Aim 1, analysis of I/LP cells in Aim 2

Name: Steve Horvath
Project Role: Co-investigator
Nearest Person Month Worked: 1
Contribution to Project: Statistical/predictive outcome analysis of I/LP signature in Aim 3

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report

What other organizations were involved as partners?
Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

N/A

9. APPENDICES: See attached 2013 PNAS article.
Prostate cancer originating in basal cells progresses to adenocarcinoma propagated by luminal-like cells

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Contributed by Owen N. Witte, November 1, 2013 (sent for review October 18, 2013)

The relationship between the cells that initiate cancer and the cancer stem-like cells that propagate tumors has been poorly defined. In a human prostate tissue transformation model, basal cells expressing the oncogenes Myc and myristoylated AKT can initiate heterogeneous tumors. Tumors contain features of acinar-type adenocarcinoma with elevated eIF4E-driven protein translation and squamous cell carcinoma marked by activated beta-catenin. Lentiviral integration site analysis revealed that alternative histological phenotypes can be clonally derived from a common cell of origin. In advanced disease, adenocarcinoma can be propagated by self-renewing tumor cells with an androgen receptor-low immature luminal phenotype in the absence of basal-like cells. These data indicate that advanced prostate adenocarcinoma initiated in basal cells can be maintained by luminal-like tumor-propagating cells. Determining the cells that maintain human prostate adenocarcinoma and the signaling pathways characterizing these tumor-propagating cells is critical for developing effective therapeutic strategies against this population.

Tumors that arise from a given tissue in the body exhibit heterogeneity with respect to their molecular alterations, biological behavior, and response to therapy (1). Such variation presents a serious challenge for clinical cancer management. In many organ sites, tumors have been classified into subtypes based on their molecular and histological features (2). Subtypes of cancer can reflect distinct states of differentiation within a given tissue, leading Visvader and coworkers to propose that different epithelial tumor subtypes can arise from transformation of distinct cells of origin with different developmental potential (3). Functional studies in the mouse mammary gland and mouse lung support this model (4, 5). However, there is limited functional evidence for such a mechanism in human epithelial cancer.

Several recent studies using mouse models have revealed that the same phenotypic cell that initiates cancer can be responsible for tumor maintenance or propagation. Lgr5+ intestinal stem cells can initiate and maintain murine intestinal adenomas (6, 7). In mouse models of skin cancer, hair follicle bulge stem cells can serve as target cells for transformation (8) and CD34+ cells resembling their normal bulge stem cell counterpart are capable of propagating the disease as a cancer stem cell population (9). Mouse models of breast cancer demonstrate that tumors can arise from the transformation of luminal cells (4), and recent studies using human tumor samples indicate that breast cancer can also be propagated by luminal-like cells (10). In most human epithelial cancers it has not been determined whether the cell types that give rise to cancer are also capable of maintaining advanced disease.

The predominant histological subtype of prostate cancer is acinar-type adenocarcinoma (11), with features of luminal secretory cells, rare neuroendocrine cells, and an absence of basal cells. A number of less common histological variants are found in prostate cancer, including small cell carcinoma and squamous cell carcinoma. Both of these variants are associated with poor prognosis, aggressive disease, and resistance to hormonal therapy (androgen deprivation and/or androgen receptor blockade) (11). Small-cell carcinoma is characterized by proliferating neuroendocrine cells and loss of p53 (12). Squamous cancers have features of basal cells and can occur either in the context of adenocarcinoma or alone as squamous cell carcinoma (11, 13, 14). Based on their different phenotypes and response to hormonal therapy, different histological variants of prostate cancer are predicted to arise from distinct cells of origin (13).

The relationship between the cells that initiate and maintain human prostate adenocarcinoma is not known. Naive human prostate basal cells can initiate acinar-type adenocarcinoma in response to oncogenic stimulation (15). Consistent with these findings, basal cells from the BPH-1 human prostate cell line can initiate human prostate cancer in response to combined estrogen and testosterone treatment (16). These collective data suggest that human prostate tumors may set aside a subset of basal cells within the tumor to ensure continuous production of malignant luminal-like cancer cells.

Human prostate cancer cells with a basal phenotype have been reported to produce luminal cancer progeny in vitro (17). Using cell lines that were originally derived from human prostate tumors, it was shown that basal cell marker CD44 enriched for tumor-propagating cells in the absence of differentiated luminal cell markers (18). A recent study demonstrates that advanced chemotherapy-resistant prostate cancer is maintained by cells lacking basal or luminal cytokeratins (19). No study has defined the role of basal or luminal-like cells isolated directly from primary human prostate cancer in tumor propagation.

In the present study, we use a tissue-regeneration model of human prostate cancer to determine whether the cells at the origin of prostate cancer are continually required to maintain the disease as tumor-propagating cells. Benign cell populations isolated from primary human prostate tissue were first tested for
their susceptibility to transformation by defined oncogenes. In the resulting tumors, cancer cell populations were further transplanted to define the cells capable of propagating the disease. Tumors driven by expression of oncogenes Myc and myristoylated/activated AKT (myrAKT) initiating in basal cells exhibit features of both adenocarcinoma and squamous cell carcinoma with different signaling pathways characteristic of each histological pattern. eIF4E-driven protein translation pathway is elevated in adenocarcinoma, whereas activation of beta-catennin is associated with squamous differentiation in experimental and clinical human prostate cancer. Using lentiviral integration site analysis, we determined that alternative histological phenotypes of prostate cancer can arise from a clonal cell of origin. Adeno- 

Results

Basal Cells Initiate Heterogeneous Human Prostate Cancer. Two of the most common alterations identified in human prostate tumors are increased expression of Myc (20) and activation of AKT, typically via loss of PTEN (21). Coexpression of Myc and phosphophorylated AKT are rarely observed in primary localized prostate cancer, but are commonly found in advanced metastatic prostate tumors (Fig. 1A). The oncogenes Myc, myrAKT, or both Myc and myrAKT were introduced into lentivirus into highly enriched populations of human prostate basal (CD45−Trop2−CD49fhiCD26−) and luminal (CD45−Trop2−CD49fhiCD26+) cells isolated by FACS. Basal and luminal epithelial cells were purified from preparations of dissociated, freshly isolated prostate tissue from six patients undergoing radical prostatectomy. Transduced human prostate epithelial cells were combined with inductive murinerogenital sinus mesenchyme (UGSM) with Matrigel and implanted s.c. into immune-deficient NOD-SCID-IL2Rγnull mice (Fig. S1A). Consistent with previous findings (15), infection of human prostate luminal-enriched cells did not result in any detectable epithelial structures after 12 wk in vivo, regardless of whether cells were transduced with a single oncogene or the combination of Myc and myrAKT. Even implantation of 100,000 luminal cells was insufficient to generate primary tumors (Fig. S1B).

When expressed in naïve human prostate basal-enriched cells, Myc or myrAKT alone gave rise to benign glands or low-grade prostatic intraepithelial neoplasia lesions with distinct p63+ basal and androgen receptor-positive (AR+) luminal layers (Fig. 1B and C). The two oncogenes dramatically synergized in human prostate basal cells to consistently generate large tumors in as little as 6 wk (Fig. 1B). Transduced basal cells were implanted in limiting numbers (103, 5 × 104, 105, and 106) in vivo to determine the number of target cells required to respond to oncogenic stimulation. As few as 10,000 basal cells were sufficient to initiate tumors in 12 wk (Fig. S1B). Metastasis was not observed in tumor-bearing mice, indicating that further genetic alterations or a longer time period may be required to observe metastatic lesions.

Regenerated tumors contained both acinar-type adenocarcinoma and squamous features (Fig. 2A). Adenocarcinoma regions were defined by high levels of expression of the luminal markers Keratin 8 (K8) and CD26, heterogeneous expression of AR, scattered chromogranin A+ neuroendocrine-like (NE) cells and an absence of basal markers Keratin 14 (K14), p63, and Keratin 5 (K5) (Fig. 2B). Conversely, squamous regions expressed basal cell markers K14, p63, and K5 and lacked cells expressing K8, CD26, AR, or chromogranin A. All tumors were confirmed to express oncogenes Myc and myrAKT and have a human origin based on staining with a pan-HLA antibody (Fig. 2B and Fig. S2). This heterogeneous tumor provides a model to investigate the relationship between different histological phenotypes within an individual cancer. In clinical prostate cancer, the squamous phenotype is rare in primary tumors. Squamous differentiation is predominantly observed in aggressive metastatic tumors that are resistant to androgen-deprivation therapy (22).

Distinct Histological Phenotypes Share a Clonal Origin. Human prostate cancer often presents as a multifocal disease where distinct cancerous lesions are present within an individual patient’s tissue. Multiple tumor foci can regularly be identified within regenerated tumors using the in vivo tissue-regeneration assay (15). We identified certain regions containing cells with adjacent squamous (K14/p63/K5) and luminal (K8/CD26) phenotypes in close proximity without defined borders (Fig. 3 A and B). These mixed regions made up an average of six foci per tumor, covering ~18% of the total tumor area (Fig. S3). These regions containing both histological phenotypes originate either from the merging of two neighboring glands of distinct histological phenotypes or from a single gland capable of multilineage differentiation. The most definitive method to distinguish these possible outcomes is lentiviral integration site analysis (23).

Tumors were initiated from naïve human prostate basal cells transduced with lentivirus, resulting in the integration of viral DNA into the genome of the target cell and all of its progeny. If both adenocarcinoma and squamous phenotypes within an individual region arise from the same transformed basal cell, they should share a set of common lentiviral integration sites. However, if distinct histological phenotypes in an individual region arise from distinct cells of origin, they should not share any common integration sites.

Laser capture microdissection was performed on neighboring adenocarcinoma and squamous phenotypes within an individual lesion (region X) and DNA was isolated separately from...
adenocarcinoma and squamous cells for lentiviral integration site analysis (Fig. 3B). DNA was also isolated from an individual lesion containing only the adenocarcinoma phenotype (region Y). In a distinct tumor, DNA was collected from neighboring adenocarcinoma and squamous phenotypes in close proximity (region Z). PCR primers specific for the viral DNA were used to extend into the genomic DNA (Fig. 3C). Illumina sequencing was performed and reads were aligned to the genome to map integration sites (Fig. 3C). Neighboring adenocarcinoma and squamous cells (region X) shared common integration sites (Fig. 3D and E), showing that they are derived from a clonal origin. In contrast, tissue taken from a distinct adenocarcinoma lesion (region Y) had entirely unique integration sites (Fig. 3D and E). Common integration sites were also found between neighboring adenocarcinoma and squamous phenotypes taken from a separate tumor (Region Z, Fig. 3 D and E). No overlapping integration sites were observed between different regions, as would be expected.

Beta-catenin signaling has been implicated in squamous differentiation and tumorigenesis in the prostate, mammary gland, and skin (9, 24, 25). In human regenerated prostate tumors, the expression of total and activated forms of beta-catenin was evaluated in both adenocarcinoma and squamous tissues. In contrast to total beta-catenin, which is expressed in both histological variants, active beta-catenin is highly expressed in squamous areas (Fig. S4). Elevated Wnt signaling in the prostate microenvironment can transform adjacent naive benign epithelium (26). Wnt ligands are elevated in prostate cancer stromal cells following treatment, leading to beta-catenin activation in the adjacent tumor cells (27). This is consistent with the presence of squamous differentiation in prostate cancer, which is rare in primary tumors but more commonly observed in late-stage prostate cancer following treatment (14, 22). High levels of activated beta-catenin could also be detected in clinical metastatic castration-resistant prostate cancer with squamous differentiation (Fig. S4).

Luminal-Like Cancer Cells Serially Propagate Adenocarcinoma in the Absence of Basal-Like Cells. Previous studies have established optimal conditions for tumorigenicity of dissociated human tumor cell preparations (28). Regenerated primary human prostate tumor cells initiated from the transformation of five distinct benign patient samples were dissociated to single cells. Human tumor cells were identified by FACS based on staining of a pan-HLA antibody (Fig. 4A). Luminal-like tumor cells were then isolated on the basis of low expression of CD49f and transplanted into secondary NSG mice.

Upon transplantation, isolated CD49f<sup>+</sup> tumor cells were capable of generating secondary tumors in recipient mice. Immunohistochemical staining on tumor-derived tissue sections identified a phenotype consistent with luminal/acinar-type adenocarcinoma (29) with a predominance of K8+ CD26+ luminal-like cells and an absence of K14+ p63+ basal-like cells (Fig. 4B). CD49f<sup>+</sup> tumor cells failed to regenerate the squamous phenotype. Secondary tumors derived from CD49f<sup>+</sup> cancer cells were almost entirely composed of a CD49f<sup>+</sup> Keratin 18+ luminal-like phenotype as analyzed by flow cytometry (Fig. 5A) and expressed oncogenes Myc and myrAKT (Fig. 5S). As few as 100 CD49f<sup>+</sup> cells taken from secondary tumors could generate tertiary tumors with a strictly adenocarcinoma phenotype (Fig. 5B). These data suggest that CD49f<sup>+</sup> tumor cells can self-renew in a unipotent manner in vivo.
CD49fhi tumor cells were also tested in transplantation assays. We found that CD49fhi tumor cells could regenerate secondary lesions exhibiting a mixture of squamous (K14+ p63+) and adenocarcinoma (K8+ CD26+) areas (Fig. S6). These findings suggest the presence of multipotent cells in the CD49fhi fraction capable of propagating both squamous and adenocarcinoma phenotypes.

In numerous tissues, high expression of Myc is associated with a block in differentiation and reprogramming to pluripotency (30). In prostate epithelium, Myc overexpression has been shown to reduce levels of AR (31) and its target prostate-specific antigen (PSA) (32). We hypothesized that high levels of Myc in Myc/myrAKT-driven tumors might cause a block in differentiation, resulting in reduced levels of AR and PSA. In primary regenerated tumors, we found low and heterogeneous expression of AR with only rare PSA+ glands (Fig. 1C and Fig. S8). Levels of AR and PSA were low in secondary and tertiary tumors upon serial transplantation (Fig. 5C and Fig. S8). These results are consistent with recent studies demonstrating that low or negative levels of AR and PSA are associated with tumor-propagating cells in prostate cancer xenografts (18, 19, 33, 34). Low levels of AR and PSA are also characteristic of DU145 (35) and PC3 (36) aggressive metastatic prostate cancer cells. In fact, expression of PSA, characteristic of clinical acinar-type adenocarcinoma, is inversely correlated with patient survival (18).

### Luminal-Like Cancer Cells Exhibit Elevated eIF4E-Driven Protein Translation

Our findings demonstrate that whereas naïve benign luminal cells are not efficient cells of origin for prostate cancer, CD49fhi tumor cells with a luminal phenotype can self-renew and maintain human prostate adenocarcinoma in the absence of CD49fhi or K14+ p63+ basal-like cells. Signaling pathways that are absent in benign luminal cells may become expressed in malignant luminal cells and contribute to their capacity to self-renew and initiate tumorigenesis. Two pathways implicated in prostate tumorigenesis are the MAPK pathway (37) and the JAK/STAT pathway (38). Myc and myrAKT-driven tumor cells exhibited low levels of phosphorylated Erk1/2, downstream of the MAPK pathway, and weak staining for phosphorylated STAT3, a readout of the JAK/STAT pathway (Fig. S9).

The protein translation factor eIF4E is downstream of the PI3K pathway and has been shown to cooperate with Myc in promoting cell growth and tumorigenesis (39). eIF4E is unable to promote translation when bound by 4EBP1. mTOR-mediated phosphorylation of 4EBP1 (p4EBP1) relieves this interaction and allows eIF4E-driven translation initiation. High levels of eIF4E and p4EBP1 are associated with poor prognosis in prostate cancer (40) and have been shown to drive tumor invasion (41).

Elevated levels of eIF4E, 4EBP1 and p4EBP1 were found in luminal-like tumor cells isolated from Myc and myrAKT-driven tumors but not in benign luminal cells or in neighboring squamous cells (Fig. 6A and B and Fig. S10). Luminal-like tumor cells also express MTA1 and Sox2 (Fig. 6A and B), two targets of eIF4E translation associated with prostate cancer progression. The self-renewal regulator Sox2 has been previously demonstrated to promote the proliferation of prostate cancer cells (42). eIF4E, p4EBP1, MTA1, and Sox2 were identified in subsets of benign basal cells but not benign luminal cells (Fig. 6A). These findings suggest that luminal-like tumor cells may acquire a limited set of self-renewal and cell-survival factors normally associated with stem-like basal cells to promote tumor propagation.

Luminal-like cancer cells were grown in vitro and treated with pharmacological inhibitors previously demonstrated to interfere with eIF4E-driven protein translation. mTOR-mediated phosphorylation of 4EBP1 can be prevented by treatment with Rapamycin, allowing nonphosphorylated 4EBP1 to bind and inhibit eIF4E. PP242 inhibits both mTORC1 and mTORC2 and has been previously demonstrated to deplete eIF4E-driven protein translation (43). Dasatinib is a Src family kinase inhibitor that does not alter 4EBP1 phosphorylation. We tested the effect of eIF4E-driven protein translation inhibition on luminal-like cancer cells isolated from Myc and myrAKT-driven tumors. Treatment with rapamycin and PP242, but not with Dasatinib, caused a reduction in MTA1 and Sox2 protein levels and depleted sphere number and size in vitro (Fig. 6C–E).
Discussion

Epithelial cancers often exhibit significant heterogeneity at the histological level (1). By engineering expression of oncogenes or loss of tumor suppressors in specific lineages via cre-lox technology, it has been reported that different histological variants of mouse breast and lung cancer can arise from distinct cells of origin (4, 5). We have used a tissue-regeneration model to determine the origins of prostate cancer heterogeneity. Based on their distinct phenotypes and biological behaviors, histological variants of prostate cancer have been proposed to arise from different cells of origin (13). Myc- and myrAKT-initiated tumors exhibit features of both acinar-type adenocarcinoma and squamous cell carcinoma. Using lentiviral integration site analysis, we demonstrate that alternative human epithelial cancer phenotypes can arise from a common clonal target cell. Activated beta-catenin is elevated in areas with squamous differentiation both in experimental and clinical prostate cancer, suggesting a role for the beta-catenin pathway either in promoting or maintaining the squamous phenotype.

Our findings that basal cells are efficient cells of origin for human prostate cancer are consistent with previous findings by our group and others (15, 16). These studies on the human disease have all used a tissue-regeneration approach to model development concomitant with tumorigenesis. Several different groups have investigated the origins of murine prostate cancer using genetically engineered mouse models in which the tumor suppressor Pten is deleted in a subset of basal or luminal cells from a young age. Depending on the genetic background of the mouse and the frequency of Pten deletion by the promoters used, there is considerable disagreement on whether basal or luminal cells generate a more proliferative, aggressive tumor that can model lethal human prostate cancer (44–46). Regardless of the assay system, studies using both mouse and human tissue confirm that transformed basal cells can generate malignant luminal progeny in vivo.

In the present study, we find that human prostate adenocarcinoma initiated by transformed basal cells can be propagated by phenotypically luminal cancer cells (Fig. 7). Such a model resembles chronic myelogenous leukemia (CML). CML is initiated in transformed hematopoietic stem cells (47, 48). Advanced disease can be maintained by granulocyte-macrophage progenitor-like cells that have gained self-renewal (49–51).

Several regulators of growth and self-renewal normally restricted to the stem-like basal cell compartment are likely to be expressed in malignant luminal cells in human prostate cancer and cooperate to promote their tumorigenic activity. In Myc- and myrAKT-driven human prostate tumors, luminal-like cancer cells exhibit elevated levels of p4EBP1 and elf4E. Luminal-like tumor cells express MTA1 and Sox2, two targets of elf4E-driven protein translation that are normally expressed in self-renewing basal cells but not benign luminal cells. These findings suggest that elf4E-driven protein translation may contribute to luminal-like tumor-propagating cell survival and self-renewal. In fact, activation of elf4E downstream of Pten deletion in murine models of prostate cancer can play an important role in luminal cell transformation (44, 46).

Methods

Full methods, including antibodies, lentiviral vectors, laser capture microdissection, DNA isolation, and nonrestrictive linear amplification-mediated (nrLAM) PCR are found in SI Methods.

**Human Tissue.** Acquisition and processing of human tissue, dissociation and isolation of distinct epithelial subsets, lentiviral transduction, and in vivo implantation have all been described in detail (52). Patient tissue is provided in a de-identified manner and is exempt from institutional review board approval. Immunohistochemistry, Western blotting, and intracellular flow cytometry were performed as previously described (15).

**Sequenial Tumor Transplantation.** Primary tumors were minced into small pieces, dissociated in 1 mg/mL type I collagenase (Invitrogen), 1 mg/mL Dispase I (BD Biosciences) for 2–4 h and further digested with 0.05% trypsin/EDTA (Invitrogen) for 5 min at 37 °C if necessary before sequential passing through 100-, 70-, and 40-μm cell strainers (BD Biosciences). Cells were stained with antibodies at 4 °C for 15–30 min and tumor cell populations were sorted on a BD FACS ARIA II into media with 50% (vol/vol) FBS (Omega Scientific). Isolated cells were sequenced by hemacytometer using trypan blue stain (Invitrogen), resuspended in 30 μl Matrigel (BD Biosciences), and implanted s.c. into NOD-SCID-LILRc(−/−) (NSG) mice with or without UGSM cells.
Animal Work. All primary, secondary, and tertiary tumors were transplanted s.c. into NOD-SCID-IL2Rγ−/− (NSG) mice. NSG mice were originally purchased from the Jackson Laboratories and were housed and bred under the care of the Division of Laboratory Animal Medicine at the University of California, Los Angeles (UCLA). Surgical castration (orchietomy) of tumor-bearing mice was performed according to protocols approved by UCLA’s Animal Research Committee.

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Supporting Information

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SI Methods

Antibodies. Antibodies used for flow cytometry included CD49F-PE, CD45-APC-Fluor 780, HLA-A/B/C-biotin, Streptavidin-APC, and Streptavidin-APC-eFluor 780 (eBiosciences); CD49F-Alexa Fluor 647 and CD26-FITC (BioLegend); and Trop2-APC (R&D Systems). Antibodies used for immunohistochemistry and Western blot included Keratin 8 and Keratin 5 (Covance); Keratin 14 and HLA-A/B/C (Abcam); p63, Erk1/2, and androgen receptor (AR) (Santa Cruz); CD26/DPP4 (LifeSpan Biosciences); chromogranin A (Dako); Myc, eIF4E, and 4EBP1 (Epitomics); Histone H3, AKT, and p4EBP1 (Thermo); pErk1/2 (T202/Y204); pSTAT3 (Y705); Sox2, MTA1, Src, and pSrc (Y416) (Cell Signaling); beta-catenin (BD Biosciences); and active beta-catenin (Millipore).

Lentiviral Vectors. The myristoylated AKT vector was previously described (1). For cloning of the Myc vector, the pMX-human-eMYC plasmid was purchased from Addgene (17966), cut with NotI, and blunted using Pfu polymerase. EcoRI linkers (Gene Link) were added to the 3′ end (former NotI site) by overnight blunt-end ligation using T4 DNA ligase (New England Biolabs). EcoRI was used to cut and release the MYC insert. The insert was gel-purified and cloned into the EcoRI sites of FU-CRW lentiviral backbone (2). Insert orientation was checked by ClaI digestion and sequenced for confirmation. The resulting plasmid is now described as FU-Myc-CRW with the construct design presented in Fig. S1.

Laser Capture Microdissection, DNA Isolation, and Nonrestrictive Linear Amplification-Mediated PCR. Paraaffin-embedded tissues were prepared on PEN membrane slides (Leica) and stained for H&E without a coverslip. Tissue corresponding to squamous and adenocarcinoma regions were isolated using the LMD7000 (Leica). Tissue was collected into a 100-μL nuclease-free PCR tube (Ambion). DNA was isolated and whole genome amplification was performed using the REPLI-g FFPE kit (Qiagen). Amplified human genomic DNA was quantitated against an absolute plasmid standard via probe-based real-time PCR using primers uc483-F (GCATGCTTCATTAACAGTGACC) and uc483-R (TTTAAATCTGAA-TGCGATGAAAGATGG) and probe FAM-uc483 (FAM-AGATCCCCACGCTCATCCGTAGTTG-Iowa Black) (3). An estimated 100–5,000 genomic equivalents of amplified DNA was used to perform nonrestrictive linear amplification PCR (4). Briefly, 100 cycles of linear amplification were performed with primer HIV3-linear (Biotin-AGTAGTGTTGCGCCCAGTCGT) Linear reactions were purified using 1.5 volumes of AMPure XP beads (Beckman Genomics) and captured onto M-280 Streptavidin Dynabeads (Invitrogen Dynal). Captured ssDNA was ligated to read 2 linker (Phos-AGATCGGGAAGAGCAGCTCATCCGTACGTCAC-3C Spacer) using CircLigase II (Epigen) in a 10 μL reaction at 65° for 2 h. PCR was performed on these beads using primer HIV3-right (AATGATACGGCGACCACGTCTGAACTCCAGTCACGT-ATCCCTCAGACCCCTTTAGTGC) and an appropriate indexed reverse primer (CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGT). PCR products were mixed and quantified by probe-based quantitative PCR and appropriate amounts were used to load Illumina v3 flow cells. Paired-end 50bp sequencing was performed on an IlluminaHiSeq 2000 instrument using a custom read 1 primer (CCCTCAGACCCCTTTAGTCA-GTGTGAAATCTCTAGCA). Reads were aligned to the hg19 build of the human genome with Bowtie (5) and alignments were condensed and annotated using custom Perl and Python scripts to locate vector integrations. Integration sites were considered present significant in a sample if they represented at least 1% of total sequence read alignments.

Inhibitors. HLA+ CD49F luminal-like cells were isolated from Myc and myrAKT-driven human prostate tumors by FACS. For drug treatment, Dasatinib (20 nM), Rapamycin (20 μM), and PP242 (10 μM or 1 μM) (all from Selleck Chemicals) or DMSO control (Sigma) were used.

Fig. S1. Schematic of naïve human prostate in vivo transformation. (A) CD45-Trop2+ epithelial cells were sorted based on CD49f and CD26 into CD49fhiCD26− basal-enriched and CD49floCD26+ luminal-enriched subsets, transduced with lentivirus carrying Myc, myristoylated/activated AKT (myrAKT), or both, combined with urogenital sinus mesenchyme cells and transplanted into NSG mice. (B) The number of grafts recovered that contain epithelial lesions per number of grafts implanted in recipient mice is indicated. At doses of 100,000 or 50,000 transformed cells, a single graft was implanted from each patient sample representing five or six individuals. At lower cell doses, duplicate grafts were implanted from two individuals.

Fig. S2. Species-specific staining for HLA-A/B/C antibody. Tissue sections of human regenerated Myc/AKT tumors and mouse prostate tissue (negative control) were stained with a human-specific pan-HLA antibody to confirm species specificity. (Scale bars, 100 μm; top left overview, 1 mm.)

Fig. S3. Quantification of stand-alone adenocarcinoma, stand-alone squamous, or mixed glands within regenerated tumors. Tissue sections representing Myc- and myrAKT-initiated tumors were subjected to quantification of the number of glands and percentage of total tumor area harboring stand-alone adenocarcinoma, stand-alone squamous, or mixed glands.
**Fig. S4.** Elevated expression of active beta-catenin in squamous cells. Representative adenocarcinoma and squamous regions from primary tumors driven by Myc and myrAKT, and a clinical metastatic castration-resistant prostate cancer (CRPC) sample with squamous differentiation were stained for H&E and antibodies against total and activated beta-catenin. (Scale bars, 50 μm.)

**Fig. S5.** Characterization of oncogene expression in histological variants present in secondary tumors initiated by CD49fhi tumor cells. Secondary tumors were stained for H&E, Myc, and myrAKT and representative regions are shown. (Scale bars, 50 μm.)

**Fig. S6.** Tumors initiated from CD49flo cells expressing Myc and myrAKT were dissociated to single cells, gated based on HLA+, and CD49fhi tumor cells were transplanted back into recipient mice. H&E-stained overview of a representative secondary tumor from 10,000 isolated CD49fhi tumor cells after 6–12 wk in vivo. Both squamous and adenocarcinoma phenotypes are represented in secondary tumors as distinguished by stains for H&E, K8, CD26, K14, and p63. (Scale bars, 50 μm.)
Fig. S7. Characterization of oncogene expression in histological variants present in secondary tumors initiated by CD49fhi tumor cells. Primary tumors initiated in naïve benign CD49fhi cells expressing Myc and myrAKT were dissociated to single cells. Isolated CD49fhi tumor cells were transplanted into recipient mice to establish secondary tumors. Secondary tumors were stained for H&E, Myc, and myrAKT and representative regions are shown. Regardless of the phenotype of secondary tumors, expression of oncogenes Myc and myrAKT was maintained. (Scale bars, 50 μm.)

Fig. S8. Low or absent expression of prostate-specific antigen (PSA) in primary, secondary, and tertiary regenerated tumors. Immunohistochemical staining for AR and PSA, a downstream target of AR signaling, in adenocarcinoma regions of regenerated tumors. Primary regenerated tumors, initiated in naïve benign CD49fhi cells, show low or negative expression of PSA except for rare PSA+ glands (Inset). PSA expression is absent from secondary/tertiary tumors maintained by CD49flo tumor cells. (Scale bars, 100 μm.)

Fig. S9. Myc- and myrAKT-driven human prostate cancer exhibits low or absent expression pSTAT3 and pErk1/2. (A) Immunohistochemical staining for pSTAT3Y705 in benign human prostate and adenocarcinoma regions of regenerated Myc and myrAKT-driven tumors (arrows denote positive nuclei). (B) Immunohistochemical staining for pErk1/2T202/Y204 in AKT- and K-RASG12V-driven mouse prostate cancer (positive control), benign human prostate and adenocarcinoma regions of regenerated Myc and myrAKT-driven tumors. (Scale bars, 50 μm.)
Fig. S10. Expression of EIF4E/4EBP1 pathway components in adenocarcinoma and squamous cells. Immunohistochemical staining for H&E, eIF4E, total 4EBP1, and phosphorylated 4EBP1 (p4EBP1) in Myc/myrAKT-driven primary tumors indicates elevated pathway activation in adenocarcinoma cells compared with neighboring squamous cells. (Scale bars, 100 μm.)