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TITLE: Targeting the Cell Surfaceome of Aggressive Neuroendocrine Prostate Cancer

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14. ABSTRACT During the course of this award, we have completed and published integrated transcriptomic and proteomic analyses of preclinical prostate cancer models that profiled cell surface antigens enriched in neuroendocrine prostate cancer (NEPC). We specifically focused on carcinoembryonic antigen-related cell adhesion molecule (CEACAM5) and L1 cell adhesion molecule (L1CAM) as two compelling targets for immune-based targeting in NEPC. We published studies using the CEACAM5 antibody-drug conjugate (ADC) labetuzumab govitecan that demonstrate activity across multiple preclinical models of NEPC including patient-derived xenograft tumors. In addition, we have investigated a chimeric antigen receptor (CAR) T cell therapy targeting L1CAM originally developed for neuroblastoma that also exhibits antigen-specific antitumor activity in NEPC models. Based on these studies, we plan to translate these findings and evaluate these therapeutic strategies for men with advanced NEPC in early-stage clinical trials.					
15. SUBJECT TERMS Neuroendocrine prostate cancer, cell surface antigens, antibody therapy, immunotherapy					
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INTRODUCTION

Background: Neuroendocrine prostate cancer (NEPC) is a common, deadly endpoint for men with late-stage metastatic prostate cancer. Up to 20% of men with lethal metastatic prostate cancers demonstrate evidence of NEPC [1, 2]. NEPC can be distinguished from conventional prostate cancer or prostate adenocarcinoma (PrAd) by histologic features, neuroendocrine (NE) marker expression, loss of either androgen receptor (AR) or downstream AR signaling, Polycomb repressive complex expression, and global methylation patterns [3-5]. Genomic studies of NEPC have also identified frequent amplification of *MYCN* and loss of both *TP53* and *RB1* [5, 6]. NEPC represents a cancer differentiation state distinct from PrAd and we hypothesize that the cell surface phenotype of these prostate cancer subtypes should reflect these differences. Furthermore, these differences in cell surface antigen expression may provide an opportunity for prostate cancer subtype-specific therapeutic targeting. The purpose of the research is to establish new NEPC models, to characterize the differential cell surface antigen profile of NEPC relative to PrAd, and to develop antibody reagents targeting novel antigens in NEPC. We believe that these studies may facilitate the development of targeted treatments for men with NEPC, for which there are no currently available FDA-approved therapeutics.

KEYWORDS

Neuroendocrine prostate cancer, cell surface antigens, antibody therapy, immunotherapy

ACCOMPLISHMENTS

To summarize the research accomplishments to date, the tasks described in the proposed Statement of Work are itemized here with a brief update for each task.

SA 1: Establish a diverse panel of NEPC cell lines from human prostate epithelial transformation (months 1-12)

Task 1: Use the human prostate organoid transformation assay with the oncogenes *MYCN* and activated *AKT1* to generate new NEPC cell lines (months 1-12) Completed during prior reporting periods. We have generated five NEPC cell lines using the human prostate transformation assay called LASCPC-01, NA1, NA2, NA3, and NA4. Characterization of these cell lines and xenografts have been performed at the level of morphology, xenograft histology, and transcriptome analysis. LASCPC-01 has been submitted to the ATCC biorepository and is currently available (ATCC CRL-3356) to the research community.

Task 2: Evaluate the effect of *p53* loss and *Rb1* loss in combination with the oncogenes *MYCN* and activated *AKT1* in initiation of NEPC (months 1-24) Completed during prior reporting periods. We have established an *in vitro* neuroendocrine (NE) transdifferentiation assay to convert AR⁺ PC (ARPC) to NEPC using a cellular reprogramming approach (**Fig. 1A**) akin to

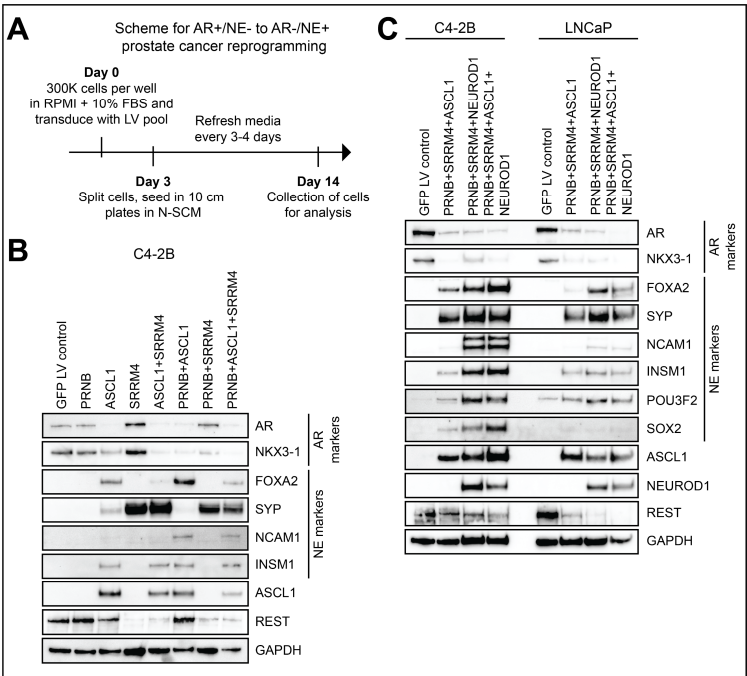


Fig. 1. Cell reprogramming of ARPC to NEPC. (A) Scheme for an *in vitro* NE transdifferentiation assay. (B-C) Immunoblots with a focus on expression of AR and NE markers. Of C4-2B and LNCaP cells collect on day 14 after being subjected to different sets of genetic perturbations in the NE transdifferentiation assay.

that used by Dr. Shinya Yamanaka's group to determine the critical factors necessary to establish induced pluripotency [7]. Briefly, we selected candidate genetic factors associated with NEPC and generated lentiviral expression vectors to introduce or disrupt these genes. The lentiviral vectors include those that encode mutant p53 R175H, a short-hairpin targeting *Rb1* (sh*Rb1*), *N-Myc*, and *Bcl-2* which we collectively refer to as PRNB. We have also included vectors expressing the splicing modulator *SRRM4* that has been shown to inactivate REST (a master negative regulator of neural gene expression) and the pioneer neural transcription factors *ASCL1* or *NEUROD1*. Enforced expression of PRNB, *SRRM4*, and *ASCL1* or *NEUROD1* in the C4-2B, LNCaP, and MDA PCa 2b in the context of this assay leads to reduced expression of *AR* and the *AR* target gene *NKX3-1* and induction of various NE differentiation markers (**Fig. 1B**).

We also determined that these cells are functionally independent of *AR* signaling by using a potent *AR*-dependent negative selection strategy (**Fig. 2A**). C4-2B cells were infected with LV encoding *AR* response elements (ARE) up-stream of a FK506 binding protein-caspase 8 (FKBP-Casp8) fusion. A clonal C4-2B

ARE-FKBP5-Casp8 line was established and transduced with either GFP LV control or the LV pool and subjected to selection with 1 nM R1881 (to induce *AR* signaling and expression of FKBP-Casp8 in *AR*⁺ cells) and AP20187 which is a synthetic FK506 analog (to activate FKBP-Casp8 and induce apoptosis). We identified no viable, adherent cells in the GFP LV control condition after 5 days of selection (**Fig. 2B-C**). In contrast, we appreciated viable cells in the LV pool condition, indicating a significant proportion of cells with functional *AR* bypass capable of surviving stringent selection against *AR* signaling.

We next performed RNA-seq on various samples from the NE transdifferentiation of C4-2B cells with the goal of determining how each factor or set of factors may contribute to the transcriptional phenotype relative to human castration-resistant prostate cancer (CRPC) and NEPC samples. Partial least squares regression (PLSR) analysis was performed to reduce highly dimensional data to features characterizing and distinguishing human CRPC and NEPC samples and subsequently applied to the C4-2B samples. Partial least squares (PLS) score plots were generated to visualize similarity/dissimilarity (**Fig. 3A**). We observed that C4-2B control samples associated with CRPC samples as anticipated and the addition of PRNB has no significant effect on the acquisition of NEPC features.

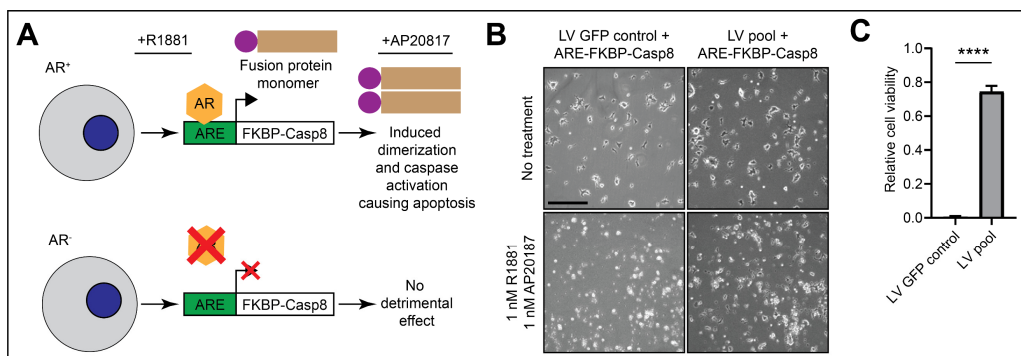


Fig. 2. Functional *AR* bypass of PC reprogrammed with LV pool. (A) Scheme for negative selection of *AR*⁺ cell populations based on *AR*-dependent expression of an inducible FKBP-Casp8 fusion protein that dimerizes and induces caspase activation and apoptosis in the presence of the FK506 analog AP20187. (B) Photomicrographs of clonal C4-2B ARE-FKBP-Casp8 transduced with either LV GFP control or LV pool (PRNB+SRRM4+ASCL1) and subjected to no treatment or treatment with R1881 and AP20187 for 5 days. Scale bar = 100 μm. (C) Relative cell viability determined by CellTiter-Glo assay of treated versus untreated groups (n=4 each) shown in panel B. ****denotes p<0.0001.

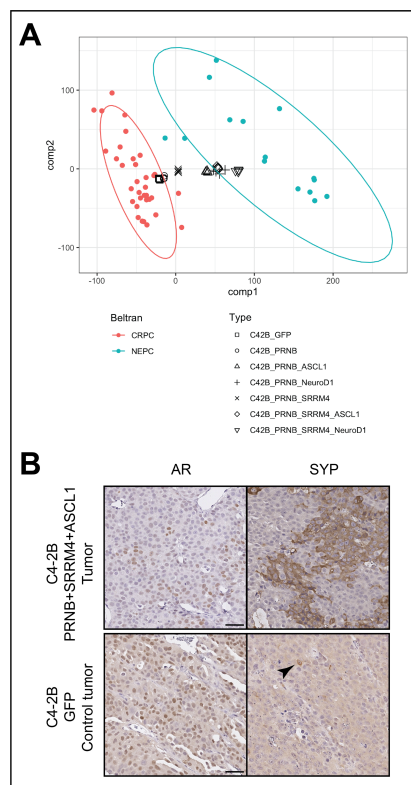


Fig. 3. Reprogramming of the C4-2B PC cell line to mixed NEPC. (A) Partial least squares regression analysis based on RNA-seq data showing C4-2B samples (black symbols) projected onto human samples (red and blue) from Beltran *H, et al. Nature Medicine. 2016*. Ellipses represent 90% confidence for CRPC or NEPC. (B) Photomicrographs showing AR of SYP expression based on IHC of tissues sections from either C4-2B GFP control tumors or C4-2B PRNB+SRRM4+ASCL1 tumors. Arrowhead denotes rare SYP expression in a cell in the C4-2B GFP control tumor section. Scale bar = 50 μm.

The addition of SRRM4 to PRNB has an intermediate effect while ASCL1 and NEUROD1, especially when introduced in combination with SRRM4 to PRNB, have a more profound effect on shifting the C4-2B cells to a NEPC phenotype.

Lastly, we have shown that these NE transdifferentiated cell line models efficiently form tumors after xenografting in NOD SCID gamma (NSG) mice. Importantly, they demonstrate phenotypes of mixed NEPC based on heterogeneous immunohistochemical (IHC) staining of AR and the NE marker synaptophysin or SYP (**Fig. 3B**). As most preclinical prostate cancer models are highly clonal, they do not reflect the tumor heterogeneity seen in advanced prostate cancer in patients. This tumor model may therefore be a very useful resource in future studies evaluating therapeutic strategies and their ability to overcome tumor heterogeneity.

SA 2: Validate candidate cell surface markers on NEPC using proteomic approaches

Task 1: High-throughput cell surface proteomic analysis of prostate cancer cell lines (months 1-14) Completed during prior reporting periods. We have published the methodology (Appendix) used to characterize cell surface proteins across a series of human prostate cancer cell lines including NEPC [8] as well as the results (Appendix) of these analyses [9]. We also contributed to a collaborative publication (Appendix) identifying L1CAM as a highly enriched cell surface protein in NEPC [10].

Task 2: Validation of candidate cell surface proteins using low-throughput proteomic techniques (months 6-18) Completed during prior reporting periods. We have completed and published a study (Appendix) confirming the expression of CEACAM5 in the University of Washington (UW) Tissue Acquisition Necropsy (TAN) cohort of tissues from lethal metastatic CRPC (mCRPC) cases [11]. These data confirm the relative frequency of CEACAM5 positivity seen in NEPC that we observed previously in another clinical case series [9].

We have optimized L1CAM IHC staining using the antibody clone UJ127 (Thermo Fisher MA5-14140) using MSKCC E1 NEPC tumor xenografts as a positive control and C4-2B tumor xenografts as negative controls (**Fig. 4A**). We have performed staining of a tissue microarray (TMA) representing multiple metastatic tissues from each of 34 patients, eight of which had histologic evidence of NEPC. We observed L1CAM staining in NEPC tissues at strong or intermediate intensity (**Fig. 4B**). We did not observe any significant staining in prostate adenocarcinoma (PrAd) tissues (**Fig. 4C**). The findings further confirm the clinical relevance of this surface protein as a potential therapeutic target.

Task 3: Prioritization of candidate NEPC cell surface proteins for therapeutic development (months 6-18) Completed during prior reporting periods. We have prioritized candidate antigens based on restricted expression in normal tissues and expression in NEPC cell lines, patient-derived xenografts, and human samples. Our leading target cell surface antigens in NEPC are CEACAM5 and L1CAM.

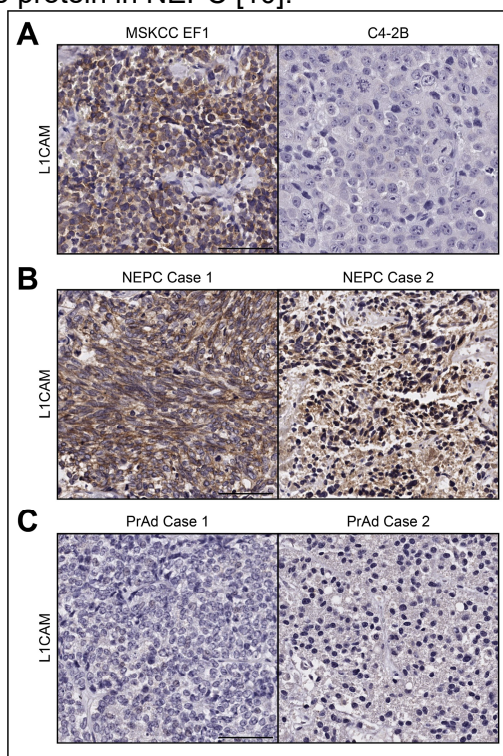


Fig. 4. L1CAM expression in lethal mCRPC tissues. (A) Optimized IHC staining of L1CAM⁺ MSKCC EF1 and L1CAM⁻ C4-2B tumor xenograft sections. L1CAM IHC staining of representative sections from (B) NEPC tissues or (C) PrAd tissues. Scale bars = 50 μ m.

SA 3: Develop novel antibody reagents targeting cell surface antigens in NEPC (months 18-48)

Task 1: Human scFv phage display library screening to identify antibody reagents (months 18-32) Completed during prior reporting periods. We previously decided to use established antibody reagents as an accelerated starting point for the development of novel immune-based therapeutic approaches.

Task 2: Validation of the specificity and performance of human monoclonal antibodies derived from phage display screening (months 18-34) Completed. We have completed analyses of binding specificity and affinity of the six fully humanized L1CAM antibody (hCE7) candidates by flow cytometry using the

IMR-32 neuroblastoma cell line with native L1CAM expression and a L1CAM knockout IMR-32 subline generated by CRISPR/Cas9 genome editing using four pooled, targeted sgRNAs (**Fig. 5A**). The six hCE7 antibodies demonstrated binding to IMR-32 across a range of concentrations (**Fig. 5B**) from which we derived dose-response curves based on antibody concentrations and mean fluorescence intensities (**Fig. 5C**). These dose-response curves were then used to calculate the half-maximal ligand binding concentration (EC50) for each hCE7 antibody candidate (**Fig. 5D**). Importantly, each of the hCE7 antibody candidates demonstrate no evidence of binding to the L1CAM knockout IMR-32 even at antibody concentrations as high as 100 nM or 300 nM (**Fig. 5E**).

Task 3: Therapeutic evaluation of monoclonal antibodies against candidate cell surface targets (months 30-40) Completed. We have completed and published a study (Appendix) demonstrating the binding specificity and genotoxicity of labetuzumab govitecan across isogenic pairs of CEACAM5- and CEACAM5⁺ prostate cancer cell lines. We have also previously shown that a second-generation 4-1BB chimeric antigen receptor (CAR) T cell therapy harboring the L1CAM CE7 single-chain variable fragment (scFv) demonstrates antigen-specific T cell activation in co-cultures with NEPC cell lines.

Task 4: Mouse studies with therapeutic monoclonal antibodies in NEPC (months 40-48) Completed. We completed and published a study (Appendix) demonstrating the antitumor activity of labetuzumab govitecan, even at reduced dose and administration schedules, in patient-derived xenografts of NEPC (LuCaP 49 and 145.1) in NSG mice [11]. In unpublished work, we have also now shown that NSG mice bearing NCI-H660 NEPC xenograft tumors treated with labetuzumab govitecan 25 mg/kg ip weekly demonstrate complete tumor responses within two weeks. Our studies do not indicate a cure but do show that if treatment is withdrawn after two weeks, tumors take much longer (up to 70 days) to recur relative to treatment with an equivalent non-targeting antibody-drug conjugate (**Fig. 6**).

In vivo studies assessing L1CAM CAR T cell therapy in MSKCC EF1 and NCI-H660 NEPC models established as tumor xenografts in NSG mice are still ongoing. These animals are being treated with either intravenous administration of human L1CAM CAR T cells or untransduced T cells with the primary readout being tumor growth inhibition based on calculated tumor volumes over time.

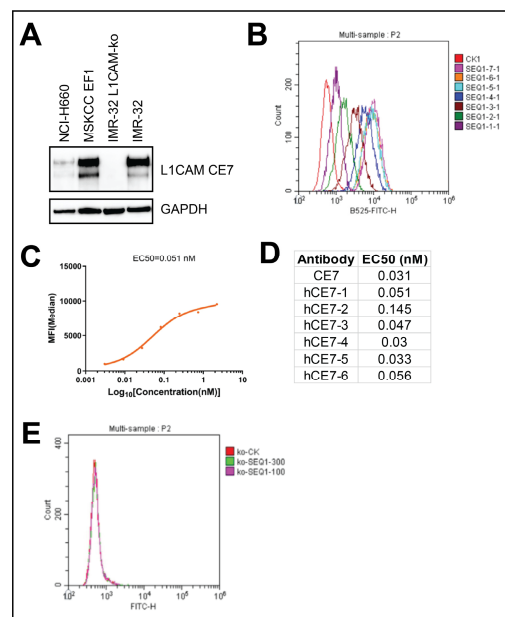


Fig. 5. Evaluating binding affinities and specificities of the hCE7 antibody candidates. (A) Immunoblot showing L1CAM expression across cell lines including the neuroblastoma cell line IMR-32 and an IMR-32 subline with L1CAM knockout (ko). (B) Flow cytometry histogram plot showing antibody binding of a dilution series of hCE7-1 antibody candidate to IMR-32 cells. (C) Graph plotting hCE7-1 antibody concentration with mean fluorescence intensity of binding to IMR-32 cells. (D) Table showing the EC50 of the parental CE7 and hCE7 candidate antibodies from flow cytometry studies using IMR-32 cells. (E) Flow cytometry histogram plot showing absence of hCE7-1 antibody binding to IMR-32 L1CAM ko cells at concentrations of 100 nM and 300 nM.

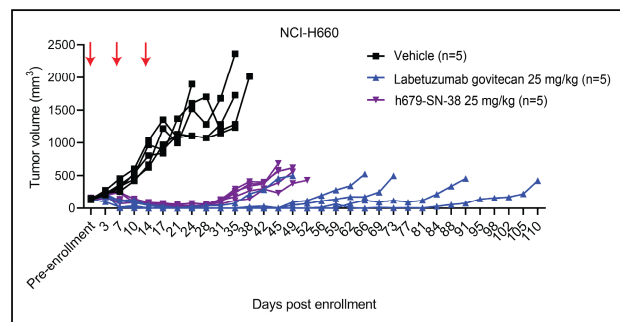


Fig. 6. *In vivo* antitumor efficacy of labetuzumab govitecan. Shown are measurements of the tumor volumes of NCI-H660 tumor xenografts in NSG mice over time after brief treatment with either vehicle, labetuzumab govitecan, or the non-targeting antibody-drug conjugate h679-SN-38.

Key Research Accomplishments:

- Established cell line models of NEPC from the human prostate epithelial transformation assay.
- Identified CEACAM5 and L1CAM as cell surface antigens enriched in NEPC.
- Conducted preclinical studies to provide a rationale for the clinical development of the CEACAM5 ADC labetuzumab govitecan for the treatment of CEACAM5⁺ NEPC.
- Continuing preclinical studies to establish the antitumor efficacy of L1CAM CAR T cell therapy for the treatment of L1CAM⁺ NEPC.
- Developed an *in vitro* NE transdifferentiation assay that recapitulates key molecular based on RNA-seq transcriptional profiling and comparison to publicly available RNA-seq gene expression data of human CRPC and NEPC samples.
- Generated a mixed NEPC tumor model using cell lines subjected to the *in vitro* NE transdifferentiation assay. The tumor heterogeneity present in this model may be valuable in therapeutic studies where understanding responses of concurrent ARPC and NEPC populations are of interest.

Opportunities for Training and Professional Development:

This final reporting period included my fourth year as an independent investigator in the Prostate Cancer Program in the Human Biology Division at Fred Hutch. My laboratory now includes two post-doctoral fellows, two graduate students, three technicians, and a staff scientist that I oversee and mentor. I continue to meet with my junior faculty mentoring committee consisting of Drs. Pete Nelson, Valeri Vasioukhin, and Bob Eisenman.

My professional development now includes multiple areas of service to the institution including as member of the Cancer Consortium Scientific Review Committee, the Faculty Diversity and Inclusion Committee, and the Proteomics Faculty Advisory Committee. I have also served as an Early Career Reviewer and ad hoc reviewer for P01 awards for the NIH and am now a Federal Advisory Committee Act (FACA) member for the Veteran's Affairs Office of Research and Development. I remain actively involved with the Prostate Cancer Foundation and the Pacific Northwest Prostate Cancer SPORE. In the past year, I received a highly prestigious NIH Director's New Innovator Award (DP2). I have also successfully applied to the NCI Experimental Therapeutics Program to develop a CAR T cell therapy for advanced prostate cancer in partnership with the NCI.

I have continued to develop positive and productive academic and industry collaborators to help translate the results of my research.

Dissemination of Results:

Our work related to this award has been published in *PNAS*, *Methods in Molecular Biology*, and *Clinical Cancer Research*. I have also had the opportunity to present these studies locally at the Fred Hutch/UW Genitourinary Oncology Seminar Series, regionally at Pacific Northwest Prostate Cancer SPORE External Advisory Board Meetings, and nationally at the Prostate Cancer Foundation Annual Scientific Meeting and the Prostate Cancer Clinical Trials Consortium Meeting.

Plan for the Next Reporting Period:

Nothing to report.

IMPACT

The discovery of CEACAM5 expression in NEPC and the redirection and demonstration of therapeutic efficacy of labetuzumab govitecan in treating preclinical models of NEPC was very compelling. This work established the basis for therapeutically targeting CEACAM5 in NEPC.

We have continued studies of L1CAM CE7 CAR T cell therapy in NEPC with the support of a Movember Foundation-Prostate Cancer Foundation Challenge Award. We have received approval and a commitment of financial backing from Bristol Myers Squibb to initiate a phase I clinical trial of L1CAM CE7 CAR T cell therapy in men with L1CAM⁺ NEPC. We anticipate that this trial will open in early 2023.

CHANGES/PROBLEMS

The project was impacted by the COVID-19 pandemic due to staffing disruptions and supply chain issues.

Based on the strength of our data, we received approval from Immunomedics, Inc. to initiate a multicenter phase I/II clinical trial of labetuzumab govitecan for men with CEACAM5⁺ NEPC. Unfortunately, shortly thereafter, Immunomedics was acquired by Gilead Sciences in October 2020. After preliminary internal review and prioritization of their therapeutic pipeline, we received notification in March 2021 that the clinical development program for labetuzumab govitecan would be on hold indefinitely. However, we received word in March 2022 that Gilead Sciences is reconsidering the development of labetuzumab govitecan including the program for clinical investigation in NEPC. We await results of the final governance meeting scheduled for June 2022.

We have also engaged with Sanofi Genzyme who has developed an alternative CEACAM5 antibody-drug conjugate called tusamitamab ravtansine which is currently in a phase III trial for metastatic non-squamous non-small cell lung cancer after having demonstrated safety and efficacy in earlier phase studies. We submitted a study concept to Sanofi Genzyme that was met with enthusiasm. We have received antibody reagents from Sanofi Genzyme to confirm the degree of expression of CEACAM5 across the UW TAN mCRPC cases. These findings will determine whether to proceed with additional preclinical studies using tusamitamab ravtansine to rationalize a clinical trial for NEPC.

PRODUCTS

Publications:

Lee JK, Bangayan NJ, Chai T, Smith BA, Pariva TE, Yun S, Vashisht A, Zhang Q, Park JW, Corey E, Huang J, Graeber TG, Wohlschlegel J, Witte ON. Systemic surfaceome profiling identifies target antigens for immune-based therapy in subtypes of advanced prostate cancer. *Proc Natl Acad Sci U S A*. 2018 May 8;115(19):E4473-E4482.

DeLucia DC and Lee JK. Identification of Cell Surface Targets for CAR T Cell Immunotherapy. *Methods Mol Biol*. 2020;2097:45-54.

Corella AN, Victoria Andrea Cabiliza Ordinio M, Coleman I, Lucas JM, Kaipainen A, Nguyen HM, Sondheim D, Brown L, True LD, Lee JK, MacPherson D, Nghiem P, Gulati R, Morrissey C, Corey E, Nelson PS. Identification of Therapeutic Vulnerabilities in Small Cell Neuroendocrine Prostate Cancer. *Clin Cancer Res*. 2020 Apr 1;26(7):1667-1677.

DeLucia DC, Cardillo TM, Ang L, Labrecque MP, Zhang A, Hopkins JE, De Sarkar N, Coleman I, da Costa RMG, Corey E, True LD, Haffner MC, Schweizer MT, Morrissey C, Nelson PS, Lee JK. Regulation of CEACAM5 and Therapeutic Efficacy of an Anti-CEACAM5-SN38 Antibody-drug Conjugate in Neuroendocrine Prostate Cancer. *Clin Cancer Res*. 2021 Feb 1;27(3):759-774.

Conference Abstracts and Presentations:

“Regulation of CEACAM5 and therapeutic efficacy of an anti-CEACAM5-SN-38 antibody-drug conjugate in neuroendocrine prostate cancer,” Pacific Northwest Prostate Cancer SPORE Scientific Symposium, Fred Hutchinson Cancer Research Center, Seattle, WA, June 27, 2020.

Corella AN, Pariva T, Coleman I, Lee JK, Nelson PS. L1CAM as a candidate therapeutic target in small-cell neuroendocrine prostate cancer. In: Abstract Book of the ESMO Targeted Anticancer Therapies Congress; March 2-4, 2020; Paris, France. *Ann Oncol*. 2020;31(Suppl 1, S8):Abstract 24P.

Kamat N, Pariva TE, Corella C, Schweizer MT, Nelson PS, Lee JK. Targeting small cell neuroendocrine prostate cancer with L1CAM chimeric antigen receptor T cell therapy. Presented at the 27th Annual PCF Scientific Retreat Virtual Poster Session; October 20-23, 2020. <https://doi.org/10.26226/morressier.5f69edb69b74b699bf38c64e>

“Regulation of CEACAM5 and therapeutic efficacy of an anti-CEACAM5-SN-38 antibody-drug conjugate in neuroendocrine prostate cancer,” Prostate Cancer Clinical Trials Consortium Principal Investigators Zoom Meeting, March 19, 2021.

“Neuroendocrine reprogramming of androgen receptor pathway active prostate cancer,” Prostate Cancer SPOREs Conference Call, September 9, 2021.

Other Products:

We have generated new cell line and tumor models of NEPC derived from parental C4-2B and LNCaP cell lines using the NE transdifferentiation assay. We are currently preparing a manuscript detailing the methodology and characterization of the resultant models. Once published, we will also make these available to investigators in the prostate cancer research community after a Material Transfer Agreement is established.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	<i>John K. Lee</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0002-6570-2180
Nearest person month worked:	8
Contribution to Project:	<i>Dr. Lee has performed data analysis and overseen the conduct of the study.</i>
Funding Support:	

Name:	<i>Tiffany Pariva</i>
Project Role:	<i>Research Technician</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	9
Contribution to Project:	<i>Ms. Pariva has conducted cell surface labeling experiments for proteomics studies and isolated RNA from cell lines for RNA-seq analysis. She has also performed IHC studies and flow cytometry.</i>
Funding Support:	

Name:	<i>Annabelle Tsao</i>
Project Role:	<i>Research Technician</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5
Contribution to Project:	<i>Ms. Tsao has cloned CARs, generated CAR T cells, and performed co-culture experiments. She has also performed antibody binding studies by flow cytometry.</i>
Funding Support:	

Name:	<i>Lauren Wiest</i>
Project Role:	<i>Research Technician</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	<i>Ms. Wiest has assisted in conducting animal experiments and colony maintenance.</i>
Funding Support:	

SPECIAL REPORTING REQUIREMENTS

Nothing to Report.

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10. Corella, A.N., et al., *Identification of Therapeutic Vulnerabilities in Small-cell Neuroendocrine Prostate Cancer*. Clin Cancer Res, 2020. **26**(7): p. 1667-1677.
11. DeLucia, D.C., et al., *Regulation of CEACAM5 and therapeutic efficacy of an anti-CEACAM5-SN38 antibody-drug conjugate in neuroendocrine prostate cancer*. Clin Cancer Res, 2020.

APPENDICES

Published manuscripts:

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Systemic surfaceome profiling identifies target antigens for immune-based therapy in subtypes of advanced prostate cancer

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Prostate cancer is a heterogeneous disease composed of divergent molecular and histologic subtypes, including prostate adenocarcinoma (PrAd) and neuroendocrine prostate cancer (NEPC). While PrAd is the major histology in prostate cancer, NEPC can evolve from PrAd as a mechanism of treatment resistance that involves a transition from an epithelial to a neurosecretory cancer phenotype. Cell surface markers are often associated with specific cell lineages and differentiation states in normal development and cancer. Here, we show that PrAd and NEPC can be broadly discriminated by cell-surface profiles based on the analysis of prostate cancer gene expression datasets. To overcome a dependence on predictions of human cell-surface genes and an assumed correlation between mRNA levels and protein expression, we integrated transcriptomic and cell-surface proteomic data generated from a panel of prostate cancer cell lines to nominate cell-surface markers associated with these cancer subtypes. FXYD3 and CEACAM5 were validated as cell-surface antigens enriched in PrAd and NEPC, respectively. Given the lack of effective treatments for NEPC, CEACAM5 appeared to be a promising target for cell-based immunotherapy. As a proof of concept, engineered chimeric antigen receptor T cells targeting CEACAM5 induced antigen-specific cytotoxicity in NEPC cell lines. Our findings demonstrate that the surfaceomes of PrAd and NEPC reflect unique cancer differentiation states and broadly represent vulnerabilities amenable to therapeutic targeting.

prostate cancer | cell surface antigens | immunotherapy

Prostate cancer is the most common non-skin cancer diagnosed in men and the second leading cause of cancer death in men (1). More than 95% of prostate cancers are diagnosed as prostate adenocarcinoma (PrAd), which is characterized by glandular epithelial architecture, expression of luminal cytokeratins (CK8 and CK18), and active androgen receptor (AR) signaling. In advanced disease, blockade of AR signaling has been the mainstay of treatment for decades, but inevitably leads to resistance in the form of castration-resistant prostate cancer (CRPC). Recent data indicate that CRPC can retain the PrAd histology or recur as a distinct subtype called neuroendocrine prostate cancer (NEPC). Recent work also indicates that a subset of CRPC assumes a double-negative (AR- and neuroendocrine-negative) phenotype that is maintained by enhanced FGF and MAPK pathway signaling (2). NEPC describes a group of neuroendocrine tumors that includes aggressive variants such as large- and small-cell carcinoma of the prostate (3). Aggressive, treatment-related NEPC evolves from PrAd in up to 20% of CRPC cases through neuroendocrine trans-

differentiation, which involves epigenetic reprogramming mediated by Polycomb proteins (4, 5) and often the loss of the tumor suppressors RB1 and TP53 (6). NEPC often exhibits an anaplastic morphology, expression of neuroendocrine markers including chromogranins and synaptophysin, loss of AR signaling, overexpression and amplification of MYCN and AURKA (7–9), and a particularly poor prognosis due to rapid and progressive metastatic dissemination.

Treatments for CRPC have expanded in recent years to include second-generation antiandrogen therapies, vaccine immunotherapy, an alpha particle-emitting radioactive agent, and additional cytotoxic chemotherapy (10). Notably, the characterization of prostate cancer cell-surface antigens like prostate-specific membrane antigen (PSMA) has spurred the development of novel diagnostic imaging and targeted therapeutic strategies. For example, PSMA-based positron emission tomography has demonstrated

Significance

Advanced prostate cancer is a deadly disease made up of multiple cancer subtypes that evolve during its natural history. Unfortunately, antibody- and cell-based therapies in development that target single tumor antigens found in conventional prostate cancer do not account for this heterogeneity. Here, we show that two major subtypes of advanced prostate cancer, prostate adenocarcinoma (PrAd) and neuroendocrine prostate cancer (NEPC), exhibit distinct cell-surface expression profiles. Integrated analysis of gene expression and cell-surface protein expression of prostate cancer nominated multiple subtype-specific cell-surface antigens. We specifically characterize FXYD3 and CEACAM5 as targets for immune-based therapies in PrAd and NEPC and provide preliminary evidence of the antigen-specific cytotoxic activity of CEACAM5-directed chimeric antigen receptor T cells in NEPC.

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high sensitivity and specificity in localizing recurrent prostate cancer (11). PSMA-targeted radioligand and chimeric antigen receptor T cell (CAR T) therapies are rapidly advancing to the clinic and represent a new generation of precision medicine for advanced prostate cancer. However, NEPC does not demonstrate uptake of PSMA radiotracers and is not expected to respond to PSMA-targeted therapies due to low-to-absent PSMA expression (12, 13). Similarly, prostate stem-cell antigen (PSCA) is another cell-surface antigen that is the focus of diagnostic and therapeutic development whose expression is heightened in the majority of PrAd, but down-regulated in NEPC (7).

The expression patterns of PSMA and PSCA in PrAd and NEPC represent a microcosm of the transcriptomic and epigenetic differences between these distinct states of prostate cancer differentiation (14). The differentiation-linked expression of cell-surface proteins has been extensively studied in lineage specification of normal tissues and in cancer (15, 16). The foremost example of this is the cluster of differentiation or classification determinant (CD) used to define stem/progenitor cells and the developmental hierarchy in normal hematopoiesis. The lineage-specific immunophenotyping of hematologic malignancies with CD molecules subsequently prompted the development of immune-based, targeted cancer therapies, including anti-CD20 antibodies (17) and recently CD19-directed CAR T (18, 19), which are transforming the treatment of B-cell malignancies. The identification of suitable target antigens in solid tumors has been limited by the lack of bona fide tissue-specific surface antigens and non-uniform antigen expression (20). New strategies to uncover target antigens and the combinatorial targeting of antigens will be needed to overcome the heterogeneity and plasticity inherent to solid tumors.

Here, we present a generalizable approach to discover cancer subtype-specific target antigens as vulnerabilities amenable to therapeutic exploitation. Our data demonstrate that divergent cancer differentiation states arising during prostate cancer progression are associated with large changes in the repertoire of expressed cell surface proteins (surfaceome). From these differences, multiple candidate antigens were nominated, and the expression of FXVD3, a FXVD domain-containing ion transport regulator, in PrAd and CEACAM5, a member of the carcinoembryonic antigen family, in NEPC were systematically validated. Lastly, we provide preliminary evidence demonstrating the feasibility of targeting CEACAM5 in NEPC with CAR T immunotherapy.

Results

Expression of Genes Encoding Cell-Surface Proteins Distinguishes PrAd and NEPC.

As PrAd and NEPC are distinct states of prostate cancer differentiation, we hypothesized that the composition of the cell surfaceome could discriminate these prostate cancer subtypes. To evaluate this possibility, a set of 7,555 putative human cell-surface proteins was bioinformatically derived (Dataset S1) from publicly available databases by using an adaptation of a published cell-surfaceome construction strategy (21). Human cell-surface proteins were predicted based on Gene Ontology (22), the TransMembrane prediction using hidden Markov models (TMHMM) (23), and glycosylphosphatidylinositol (GPI)-anchored protein annotations (Fig. 1A). Unsupervised hierarchical clustering of human prostate cancer RNA sequencing (RNA-seq) datasets (7, 14) and a cDNA microarray dataset of patient-derived xenografts (PDXs) (24) based on expression of the cell-surface gene set consistently differentiated PrAd and NEPC samples (Fig. 1B and SI Appendix, Fig. S1A and B).

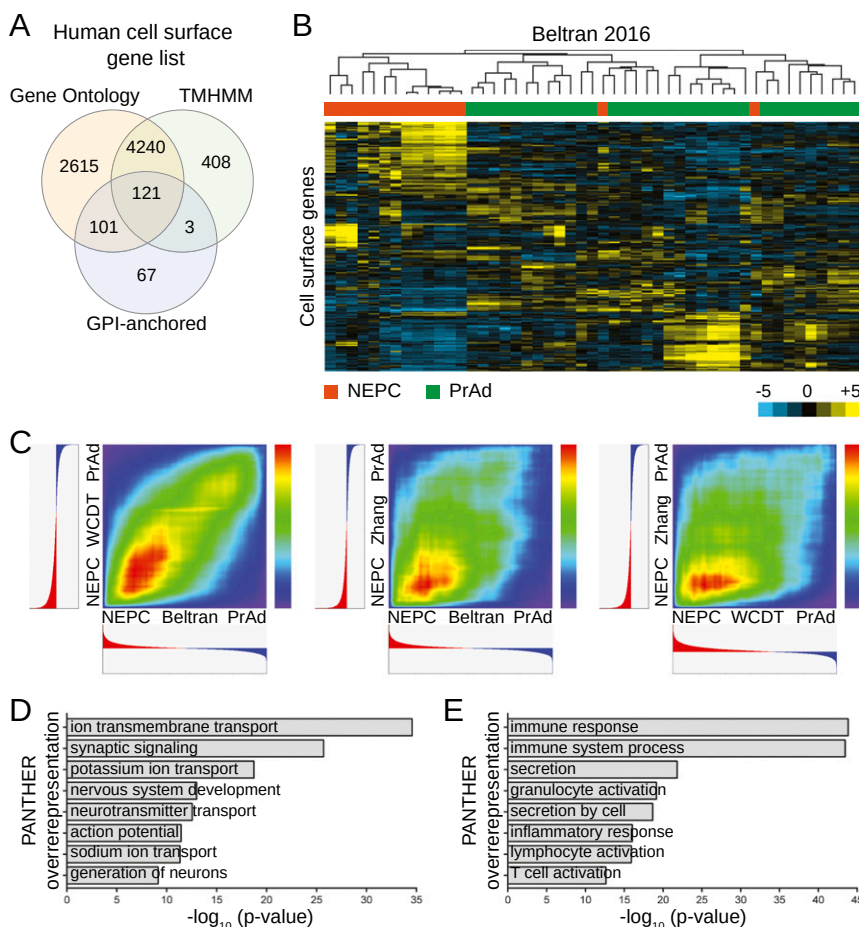


Fig. 1. Expression of genes encoding human cell-surface proteins distinguishes prostate cancer subtypes. (A) Venn diagram of a putative human cell-surface gene set bioinformatically constructed from the analysis of Gene Ontology, TMHMM, and GPI-anchored protein databases. (B) Heatmap demonstrating unsupervised hierarchical clustering of CRPC samples from the Beltran 2016 RNA-seq dataset based on the expression of cell-surface genes. Color bar represents a log₂ scale. NEPC samples are labeled in orange and PrAd samples in green. (C) RRHO heatmaps showing rank overlap of differentially expressed cell-surface genes across NEPC and PrAd samples in pairwise comparisons of the Beltran 2016, SU2C/AACR/PCF West Coast Dream Team (WCdT), and Zhang 2015 gene expression datasets. (D and E) Gene enrichment analysis from PANTHER overrepresentation testing of cell-surface genes differentially expressed more than fourfold in NEPC relative to PrAd (D) and PrAd relative to NEPC (E) in the Beltran 2016 dataset.

To determine whether the cell-surface phenotype was more highly conserved in PrAd or NEPC, we used rank–rank hypergeometric overlap (RRHO) analysis (25) to compare the ranked differential expression of genes encoding cell-surface proteins (heretofore called “cell surface genes”) between prostate cancer subtypes in multiple datasets [WCDT from the Stand Up To Cancer/American Association for Cancer Research/Prostate Cancer Foundation West Coast Dream Team (26), Beltran 2016 (14), and Zhang 2015 (24)]. This rank-based methodology circumvents the complications of normalization for specific sample preparations and analysis platforms, enabling facile comparisons of gene expression across defined classes of prostate cancer in published datasets. Pairwise evaluation of the datasets revealed higher rank correlation of cell-surface genes enriched in NEPC (Fig. 1C), supporting stronger homogeneity of cell-surface phenotypes in NEPC than in PrAd.

Differential expression analysis of the Beltran 2016 dataset showed that the expression of 330 cell-surface genes was enriched fourfold or more in the PrAd samples, while expression of 438 cell-surface genes was similarly enriched in the NEPC samples. PANTHER (Protein ANnotation THrough Evolutionary Relationship) analysis was performed to compare differentially expressed genes to a reference gene list to identify enriched molecular functions and biological processes (27). PANTHER overrepresentation testing of the cell-surface genes enriched in NEPC in the Beltran 2016 dataset identified gene ontologies related to neural functions, including synaptic signaling, nervous system development, and neurotransmitter transport (Fig. 1D). In contrast, analysis of cell-surface genes enriched in PrAd from the same dataset revealed biological processes involving secretion, immune response, and inflammatory response (Fig. 1E). The results highlight substantial differences in cell-surface antigen expression linked to the cancer differentiation states of NEPC and PrAd.

Identification of Candidate Prostate Cancer Cell-Surface Antigens by Transcriptomic Analysis. We next assembled a diverse panel of human prostate cancer cell lines to further characterize cell-surface antigens in the PrAd and NEPC subtypes. The panel included established lines including CWR22Rv1, LNCaP, NE1.3 (28), DU145, NCI-H660, and LASCPC-01 (8), as well as two developed cell lines named NB120914 and MSKCC EF1. NB120914 was initiated from an intraoperative biopsy of a metastatic castration-resistant PrAd involving the femur. While the original tumor showed a luminal phenotype with CK8 and AR expression, the resultant PDX tumor and subsequent cell-line xenograft tumors lacked expression of both luminal and neuroendocrine markers, indicative of the development of double-negative (AR- and neuroendocrine-negative) prostate cancer (*SI Appendix, Fig. S2 A, B, and D*) (2). MSKCC EF1 was adapted from 3D organoid culture (29) to suspension culture and formed xenograft tumors marked by NEPC histology, absence of AR, and expression of the neuroendocrine marker synaptophysin and p63 (*SI Appendix, Fig. S2 C and E*).

RNA-seq gene-expression analysis revealed significant heterogeneity in expression of androgen-regulated genes, neuroendocrine markers, and epithelial markers in the cell line panel (Fig. 2A), demonstrating a diverse range of molecular phenotypes. Unsupervised hierarchical clustering analysis of the prostate cancer cell lines based on the expression of cell-surface genes yielded clusters of NEPC lines (MSKCC EF1 and NCI-H660), AR-negative PrAd lines (DU145 and LASCPC-01 marked by mixed NEPC and PrAd phenotypes and NB120914), and AR-positive PrAd lines (CWR22Rv1, LNCaP, and the LNCaP-derivative NE1.3) (Fig. 2B). Differential cell-surface gene expression was evaluated in the PrAd and NEPC cell lines and identified the established PrAd markers PSMA and FOLH1 (PSMA), as well as the NEPC marker neural cell adhesion molecule 1 (NCAM1) (Fig. 2C).

We then integrated multiple prostate cancer gene expression datasets to identify differentially expressed PrAd- and NEPC-specific cell-surface markers that are conserved in prostate cancer

cell lines, PDXs, and patient tumors. For each of the datasets (WCDT, Beltran 2016, Zhang 2015 LuCaP PDXs, Zhang 2015 metastatic CRPC, and prostate cancer cell line panel), we performed rank overlap analysis by ranking the top 500 differentially expressed PrAd or NEPC cell-surface genes from each dataset and evaluating the overlap of these genes across datasets. A total of 21 genes were enriched in PrAd samples in all datasets, including well-established biomarkers and target antigens for prostate cancer therapeutics such as FOLH1 (PSMA), TACSTD2 (Trop2), and STEAP1 (Fig. 2D and *Dataset S2*). A total of 56 genes were commonly identified in NEPC samples across the datasets (*Dataset S2*). Notable from this set of genes were RET, DLL3, and SEZ6 that have been identified as disease markers in neuroendocrine cancers, including medullary thyroid cancer, small- and large-cell lung cancer, and malignant pheochromocytoma (30–32).

Prioritization of High-Confidence Cell-Surface Markers by Integrated Proteomic and Transcriptomic Analysis. While transcriptomic analysis of the prostate cancer subsets for the identification of cell-surface antigens appeared informative, we needed to overcome (i) any inaccuracy of bioinformatic predictions of human cell-surface genes and (ii) the potential for discordance between mRNA levels and protein expression (33). We therefore directly profiled the surfaceomes of the prostate cancer cell-line panel by incubation with a membrane-impermeable biotin to label primary amines on extracellular domains of cell-surface proteins, cell lysis, streptavidin-affinity purification to enrich for biotinylated proteins, trypsin digestion, and quantitative proteomic mass spectrometry (34, 35). Then, 1,080 total proteins were identified, with 45.6% annotated for plasma membrane localization by Gene Ontology (Fig. 3A). Unsupervised clustering of the cell lines based on cell-surface proteomics showed two major clusters with NEPC and AR-negative PrAd lines segregated from AR-positive PrAd lines (Fig. 3A). To evaluate the relative concordance of the cell-surface gene expression and proteomic data from the cell lines, we compared the expression levels of known prostate cancer surface markers including FOLH1 (PSMA), STEAP1, and NCAM1. The gene- and protein-level expression of these markers in the cell-line panel were similar (Fig. 3B), with minor exceptions likely explained by protein-specific availability of primary amines for biotinylation, limitations in the sensitivity of mass spectrometry, and inherent variation in the abundance of proteins and their cognate mRNAs.

As a strategy to identify high-confidence markers in the NEPC and PrAd lines, we integrated the transcriptomic and proteomic data by RRHO analysis to prioritize surface markers that are enriched at the mRNA level and exhibit differential protein expression. Overall, the rank correlation between differentially expressed cell-surface genes and proteins was highest in the NEPC lines (Fig. 3C). We generated a composite rank by arbitrarily assigning equal weights to the proteomics and transcriptomics ranks of differentially expressed proteins or genes for each prostate cancer subset (Fig. 3D). Of the candidates with high composite ranks, the PrAd-specific expression of STEAP1, FXYD3, and FOLH1 (PSMA) and the NEPC-specific expression of NCAM1, SNAP25, and CEACAM5 were validated by immunoblot (Fig. 4A) and immunohistochemistry (IHC) (Fig. 4B) of prostate cancer cell lines and xenografts. Importantly, the expression patterns of these antigens in published human prostate cancer RNA-seq datasets mirrored the subtype-enriched expression observed in the cell-line panel (*SI Appendix, Fig. S3*). Flow cytometry confirmed the surface-protein expression of STEAP1 and FXYD3 on the LNCaP PrAd line, but not on the NCI-H660 NEPC line (Fig. 4C). Conversely, surface-protein expression of NCAM1 and CEACAM5 were found on NCI-H660, but not on LNCaP.

Validation of FXYD3 as a Tumor Antigen in PrAd. FXYD3 belongs to the FXYD family of regulators of Na⁺/K⁺ ATPases that contain a 35-amino acid signature sequence domain beginning with PFXYD (36). FXYD3 has previously been found to be overexpressed in a variety of cancers, including those of the breast, stomach, and pancreas (37–39). FXYD3 was strongly enriched in

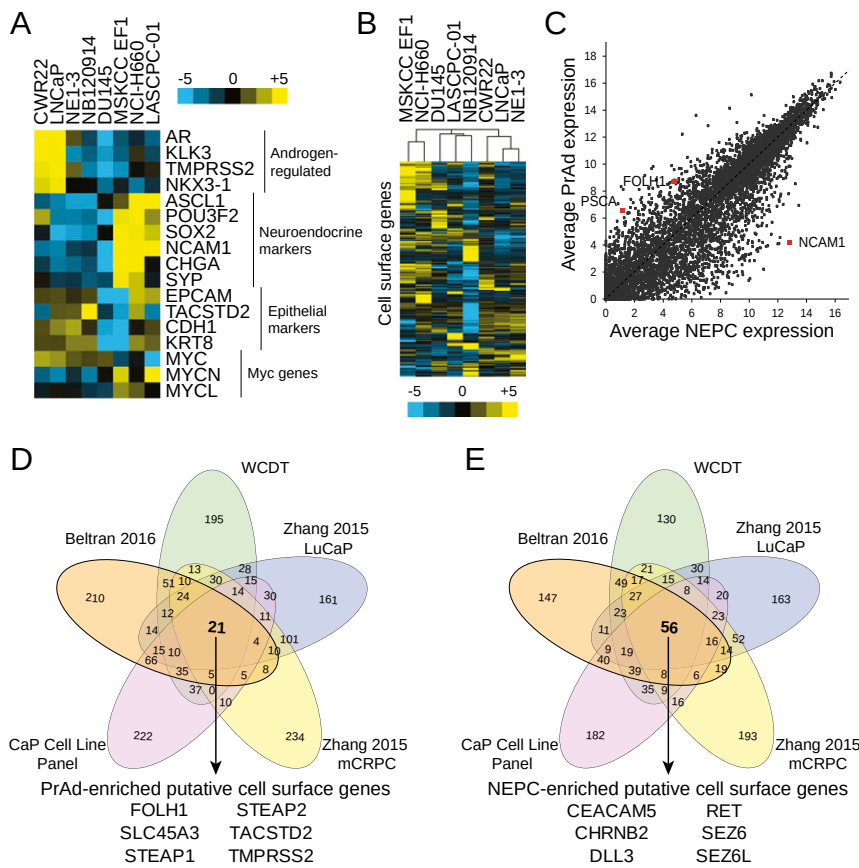


Fig. 2. Transcriptomic analysis identifies candidate PrAd- and NEPC-specific cell surface markers. (A) Heatmap of the gene expression of select androgen-regulated, neuroendocrine, and epithelial markers and Myc genes based on RNA-seq of a diverse panel of human prostate cancer cell lines. Color bar represents a log₂ scale. (B) Heatmap showing unsupervised hierarchical clustering of human prostate cancer cell lines based on the expression of cell-surface genes. (C) Plot of average expression of genes in NEPC vs. PrAd prostate cancer cell lines with select markers (NCAM1, FOLH1, and PSCA) highlighted. Gene expression is shown in log₂ scale. (D and E) Venn diagrams showing rank overlap of the top 500 differentially expressed cell-surface genes in PrAd relative to NEPC (D) and NEPC relative to PrAd (E) in each of five gene-expression datasets [prostate cancer (CaP) cell line panel, Beltran 2016, WCDT, Zhang 2015 LuCaP xenografts, and Zhang 2015 metastatic CRPC (mCRPC) samples]. Listed are the genes identified from rank overlap analysis that are enriched in all of the datasets evaluated.

the integrated transcriptomic and proteomic analysis in PrAd cell lines, but not as highly ranked by the transcriptome-based rank overlap analysis of the diverse prostate cancer datasets.

To evaluate FXYD3 expression in prostate cancer, we performed FXYD3 IHC on a tissue microarray of benign prostate samples as well as treatment-naïve primary Gleason grade 1–5 PrAd and metastatic PrAd. All 14 benign prostate tissues and 34 PrAd samples demonstrated FXYD3 expression (Fig. 5A), with the majority demonstrating moderate to strong staining specific to the normal and cancerous prostate epithelial cells (Fig. 5A and C). FXYD3 IHC was also performed on a series of small cell NEPC tissues archived at the University of California, Los Angeles (UCLA) (SI Appendix, Fig. S4). We found that in several samples with mixed PrAd and small-cell NEPC, FXYD3 appeared to be more highly expressed in the PrAd components of the tumors (Fig. 5B). Quantitation of the FXYD3 IHC scores in all evaluated samples showed that FXYD3 protein expression was reduced on average in small-cell NEPC relative to benign prostate and PrAd (Fig. 5C).

Evaluation of the NIH Genotype-Tissue Expression (GTEx) database showed that FXYD3 gene expression in human males is expressed in a variety of tissues including the skin, esophagus, stomach, small intestine, colon, bladder, and prostate (SI Appendix, Fig. S5A) (40). Congruent with the gene-expression data, IHC of a normal human tissue microarray demonstrated FXYD3 protein expression primarily in these organs (SI Appendix, Fig. S5B). The broad range of normal tissue expression would indicate that FXYD3 may not be suitable as a single target for highly potent cell-based immunotherapy due to the potential for on-target off-tumor toxicities. However, whether FXYD3 may be amenable to targeting by monoclonal antibodies or antibody drug conjugates (ADCs) is unknown, as dose-limiting off-tumor toxicity has not necessarily correlated with normal tissue expression (41).

Validation of CEACAM5 as a Target Antigen in NEPC. CEACAM5 was identified as a candidate NEPC target antigen by both transcriptomic analysis of diverse prostate cancer datasets (Fig. 2E) and by integrated transcriptomic and proteomic analysis of the prostate cancer cell lines (Fig. 3C). In support of these findings, coexpression analysis of the Beltran 2016 dataset identified that the gene expression of CEACAM5 is highly correlated with the neuroendocrine marker chromogranin A (SI Appendix, Fig. S3B) (14). A similar, strong correlation was also found between CEACAM5 and the proneural pioneer transcription factor and high-grade neuroendocrine carcinoma marker ASCL1 in a metastatic prostate cancer gene-expression dataset (SI Appendix, Fig. S3C) (42, 43). CEACAM5 (CEA or carcinoembryonic antigen) is a glycoposphatidylinositol-anchored membrane protein and established tumor antigen whose expression has primarily been associated with adenocarcinomas of the colon, rectum, and pancreas. Despite case reports of detectable serum CEA in rare patients with advanced prostate cancer, a systematic study of CEACAM5 IHC in prostate tumors identified no expression in both primary and metastatic samples (44).

To verify protein expression of CEACAM5 in NEPC, we performed IHC on a prostate cancer tissue microarray of the LuCaP series of PDX models (45). While the 13 androgen-sensitive PrAd PDXs and 9 castration-resistant PrAd PDXs evaluated did not demonstrate CEACAM5 expression, all 4 NEPC PDXs exhibited moderate or strong CEACAM5 staining localized to the plasma membrane (Fig. 6A). We extended the IHC evaluation of CEACAM5 expression to a series of small-cell NEPC tissues archived at UCLA and a tissue microarray of benign prostate samples, as well as treatment-naïve primary Gleason grade 1–5 PrAd and metastatic PrAd. Eleven of 18 (61.1%) of the small-cell NEPC tissues stained for CEACAM5 in the plasma membrane (Fig. 6B and C and SI Appendix, Fig. S6). In contrast, all 14 benign prostate tissues and 34 PrAd samples encompassing primary and metastatic

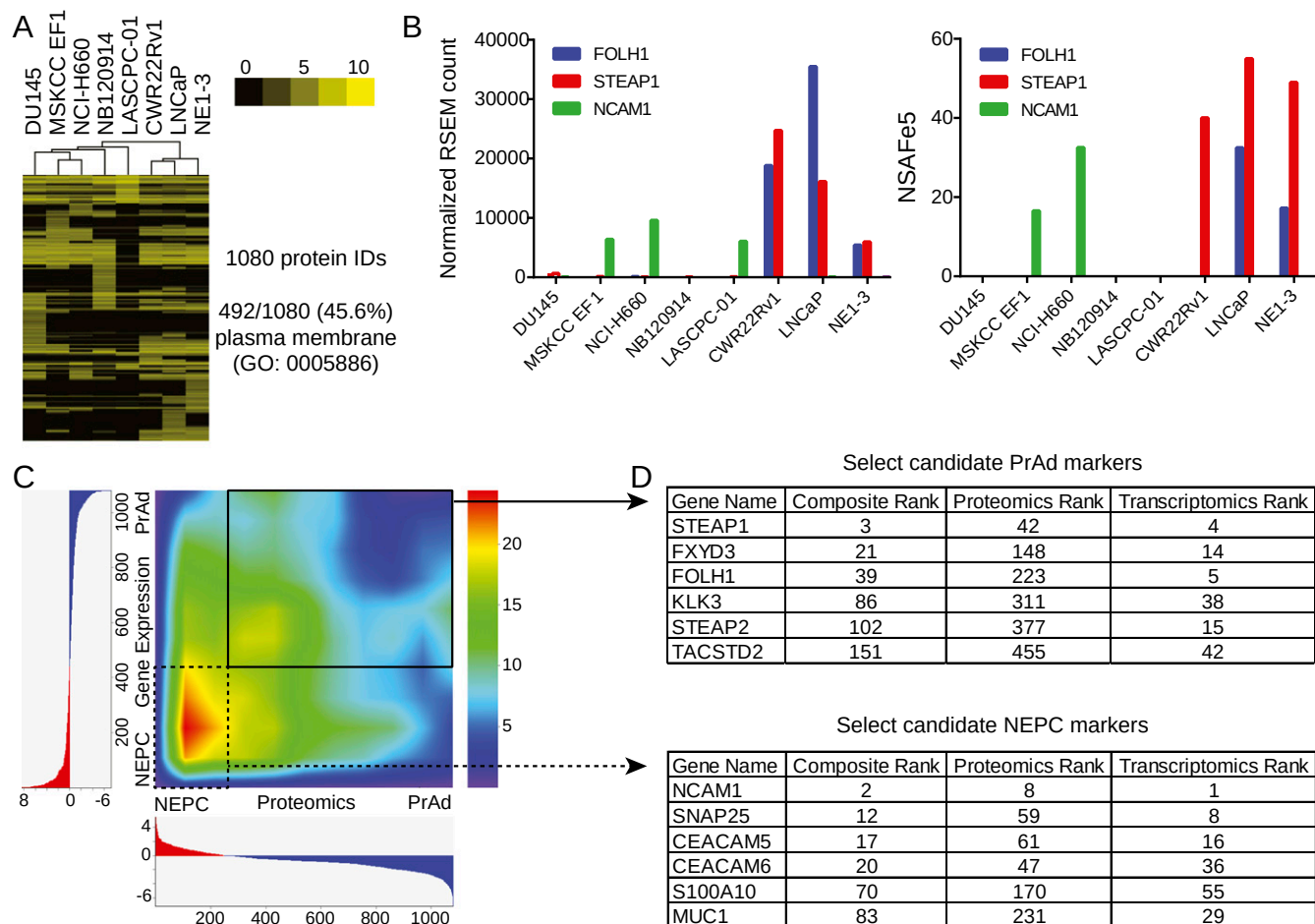


Fig. 3. Integration of cell-surface proteomics with transcriptomics nominates high-confidence PrAd and NEPC cell surface proteins. (A) Heatmap displaying unsupervised hierarchical clustering of prostate cancer cell lines based on the expression of cell-surface proteins identified from cell-surface proteomics. Depicted are normalized protein abundance values (NSAF5). Color bar represents a $\log_2$ scale. GO, Gene Ontology. (B) Comparison of the normalized RSEM gene expression counts and NSAF5 values for the cell-surface markers FOLH1, STEAP1, and NCAM1 in each of the prostate cancer cell lines. (C) Rank-rank hypergeometric heatmap showing rank overlap of differentially expressed cell-surface proteins vs. cell-surface genes identified from cell-surface proteomics and RNA-seq gene-expression analysis of the prostate cancer cell line panel. (D) Select markers demonstrating concordantly enriched protein and gene expression in the PrAd or NEPC cell lines are shown with their associated composite, proteomics, and transcriptomics ranks.

tissues were devoid of CEACAM5 immunoreactivity (*SI Appendix, Fig. S7*). These IHC validation studies indicate that CEACAM5 expression appears to be prevalent in and specific to the NEPC subtype of prostate cancer.

Therapeutic Targeting of CEACAM5 in NEPC. CEACAM5 is an antigen that is the active focus of therapeutic development in colorectal cancer with ADCs and CAR T cells (46, 47). Given our findings, we sought to examine the potential for CEACAM5-targeted therapy in NEPC. We first explored safety implications by examining the systemic expression of CEACAM5 in normal human tissues at the mRNA and protein levels. Evaluation of the NIH GTEx database showed that CEACAM5 gene expression in men is limited to the colon, esophagus, and small intestine (*SI Appendix, Fig. S84*) (40). A previous study of adoptive cell therapy with T cells engineered to express a high-affinity murine T cell receptor (TCR) targeting CEACAM5 in patients with metastatic colorectal cancer reported tumor regression, but also severe, transient colitis (48). However, data from a phase I trial of CEA-directed CAR T cell immunotherapy in CEA-positive metastatic colorectal cancers have indicated that CEA CAR T cell therapy may be well tolerated without evidence of colitis, even at high doses (47). In concordance with gene-expression data from the GTEx database, immunoblot

analysis of a range of human tissue lysates from vital organs revealed absence of CEACAM5 protein expression in the brain, heart, kidney, liver, and lung (Fig. 4A). In addition, IHC of a normal human tissue microarray demonstrated CEACAM5 expression limited to the luminal lining of the colon and rectum in men (*SI Appendix, Fig. S8B*).

Given the relatively restricted systemic expression of CEACAM5 and the highly aggressive clinical nature of NEPC, we chose to engineer CARs targeting CEACAM5 to leverage both antigen specificity and cytotoxic potency of this technology. We generated two lentiviral CEACAM5 CAR constructs encoding a single chain variable fragment (scFv) derived from labetuzumab (49), hinge/spacer, CD28 transmembrane domain, CD28 costimulatory domain, and CD3 ζ activation domain (Fig. 7A). The CEACAM5 CARs differed based on the presence of either a short spacer (IgG4 hinge) or long spacer (IgG4 hinge and CH2+CH3 spacer). We transduced T cells expanded from human peripheral blood mononuclear cells (PBMCs) with the CAR constructs and performed coculture assays with the target NEPC cell lines MSKCC EF1 (CEACAM5-negative; Fig. 4A and B), MSKCC EF1-CEACAM5 (engineered to express CEACAM5), and NCI-H660 (CEACAM5-positive; Fig. 4) at a fixed effector-to-target ratio of 1:1. Analysis of the supernatant at 12 and 24 h by IFN- γ (IFN- γ) ELISA revealed enhanced antigen-specific IFN- γ release associated with

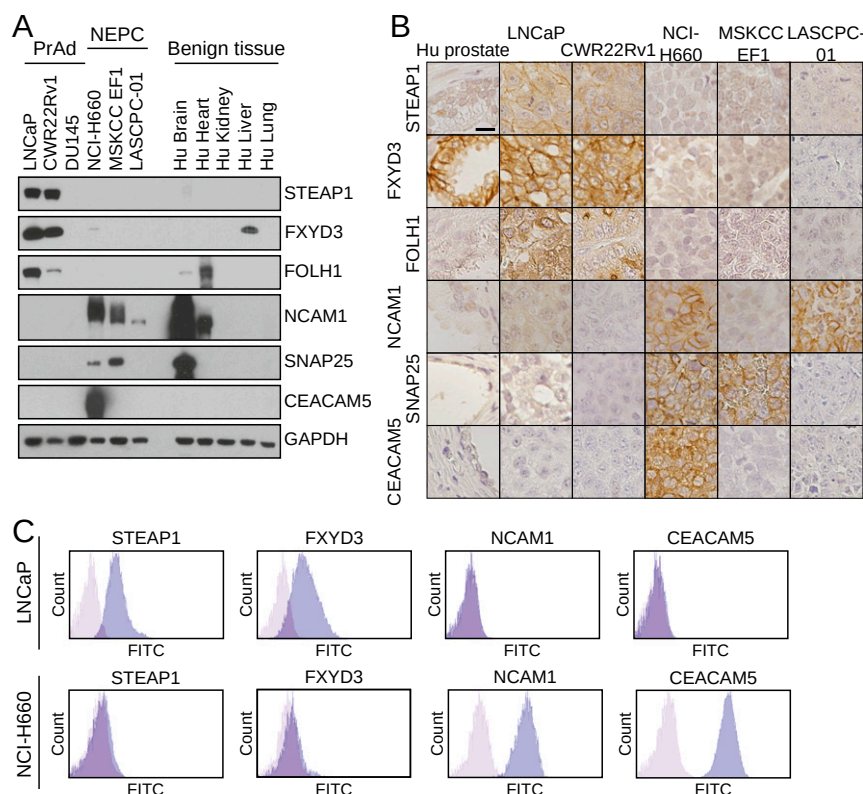


Fig. 4. Validation of candidate prostate cancer subtype-specific cell-surface antigens. (A) Immunoblot analysis of select PrAd (LNCaP, CWR22Rv1, and DU145) and NEPC (NCI-H660, MSKCC EF1, and LASCPC-01) cell lines as well as benign human tissues (brain, heart, kidney, liver, and lung) with antibodies against STEAP1, FXYD3, FOLH1, NCAM1, SNAP25, CEACAM5, and GAPDH as a loading control. (B) Human prostate tissue (Hu prostate) or prostate cancer cell line (LNCaP, CWR22Rv1, NCI-H660, MSKCC EF1, and LASCPC-01) xenograft sections after immunohistochemical staining with antibodies for the candidate antigens from A. (Scale bar, 25 μ m.) (C) Flow cytometry histogram plots of the PrAd cell line LNCaP and the NEPC cell line NCI-H660 stained with antibodies against STEAP1, FXYD3, NCAM1, and CEACAM5.

the long spacer CEACAM5 CAR (Fig. 7B). As the A3B3 domain of CEACAM5 recognized by the scFv is proximal to the membrane, a longer spacer may be necessary for optimal binding and T cell activation (49, 50).

To quantify cytotoxicity, we performed coculture assays in an Incucyte ZOOM (51), a live-cell imaging and analysis system allowing for direct enumeration of effector and target cells based on bright-field and fluorescence imaging. Varying effector-to-target ratios of T cells transduced with the long spacer CEACAM5 CAR and either MSKCC EF1 (CEACAM5-negative) or NCI-H660 (CEACAM5-positive) target NEPC cell lines engineered to express green fluorescent protein (GFP) were cocultured. Target cell counts were calculated and plotted to show relative target cell viability over time in coculture with effector cells. Coculture of long spacer CEACAM5 CAR-transduced T cells with NCI-H660 led to >80–90% cell kill by 48 h at effector-to-target ratios of 1:1 and 2:1 (Fig. 7C). In contrast, coculture with the MSKCC EF1 caused a minor reduction in target cell viability by 48 h, which may be related to low levels of CEACAM5 expression in the MSKCC EF1 NEPC cell line. Similar coculture studies were also performed with the PrAd cell line DU145 (CEACAM5-negative) and DU145-CEACAM5 (engineered to express CEACAM5). Long spacer CEACAM5 CAR-transduced T cells had negligible effects on the DU145 cells but induced significant T cell activation and target cell death when cocultured with DU145-CEACAM5 cells (*SI Appendix, Fig. S9 A and B*). These findings provide preliminary support for CEACAM5 as a promising target antigen for further therapeutic development in NEPC.

Discussion

Therapeutic development for advanced prostate cancer has increased significantly over the last decade. Both antibody- and cell-based immune treatment strategies are now poised to advance to the clinic, as monoclonal antibodies, ADCs, and CAR T cells are under clinical investigation. Most of these prospective therapies are focused on PSMA and PSCA as target antigens in CRPC. However, the heterogeneity of CRPC and the potential for treatment-induced plasticity (6, 52, 53) indicate that agents targeting only PSMA and PSCA are unlikely to eradicate the disease. An additional complication is the paucity of cancer-specific antigens that are not expressed in normal tissues (54). Sequential or combinatorial treatment strategies targeting distinct antigens while optimizing safety at various stages of disease progression will likely be necessary (55). To this end, we have characterized the surfaceome of advanced prostate cancer and generated a collection of putative target antigens using a discovery pipeline based on mRNA and cell-surface protein expression data. These studies are relevant and timely with the intent of expanding the development of targeted biologic therapies for advanced prostate cancer.

We have identified significant biological differences between the PrAd and NEPC subsets based on cell-surface protein profiling. Our data indicate that PrAd and NEPC express distinct cell-surface markers that mirror their respective glandular epithelial and neuroendocrine cancer differentiation states. Global cell-surface gene-expression analysis of these subsets across multiple published prostate cancer datasets clearly indicate that the surface phenotype of NEPC is more conserved than that of PrAd. This finding is consistent with the observed heterogeneity of PrAd that demonstrates a broad spectrum of histologic

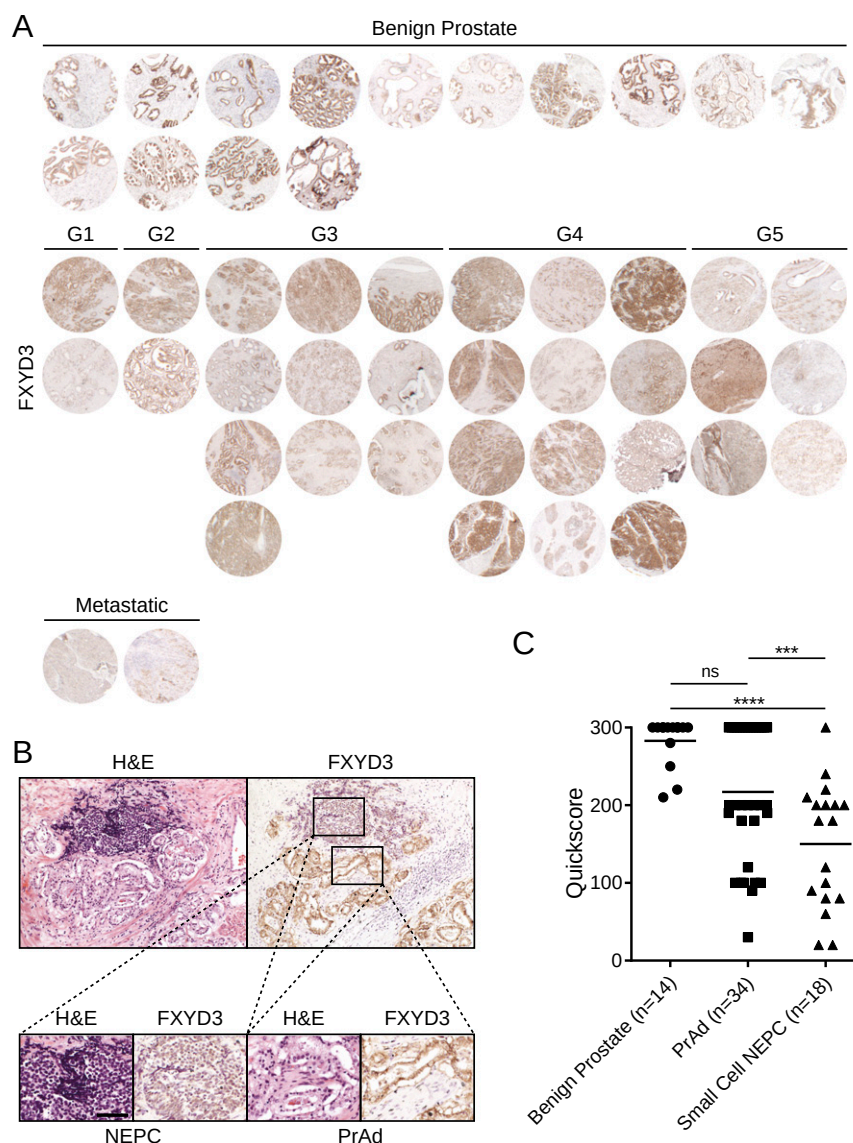


Fig. 5. FXYD3 is a cell-surface antigen whose expression is enriched in benign prostate epithelial cells and PrAd. (A) FXYD3 immunohistochemical stains of benign prostate tissues ($n = 14$), primary Gleason grade 1–5 PrAd tissues ($n = 32$), and metastatic PrAd samples ($n = 2$). (B) H&E and FXYD3 immunohistochemical stains of a section of mixed PrAd and NEPC. (Scale bar, 200 μ m.) (C) Quantitation of FXYD3 IHC in benign prostate tissues ($n = 14$), PrAd ($n = 34$), and small-cell NEPC samples ($n = 18$) by Quickscore (intensity $\times$ percentage of positive cells; maximum score is 300). ns, nonsignificance. $**P < 0.01$; $****P < 0.0001$ (by one-way ANOVA statistical analysis).

features, molecular subtypes, and clinical behaviors. On the other hand, the cell-surface profile of NEPC appears relatively homogeneous, suggesting that transdifferentiation to NEPC may represent a phenotypically constraining evolutionary path.

To nominate specific target antigens in prostate cancer as potential immunotherapeutic targets for further validation, cell-surface gene-expression and proteomics data were integrated from a diverse panel of prostate cancer cell lines. However, a pitfall of this approach is that mRNA abundance does not necessarily correlate with protein abundance (56), likely due to posttranscriptional and post-translational modifications affecting stability. In the future, technological improvements in ultrasensitive quantitative mass spectrometry in proteomics may obviate the need to consider mRNA data and enable global surfaceome analysis for target antigen discovery directly from biopsy specimens. Another limitation in the validation and therapeutic translation of candidate target antigens is the availability of specific immunoaffinity reagents against extracellular protein domains. Large-scale efforts to characterize the expression

of all human proteins in both normal and cancerous cells and tissues have been fraught with issues of data reliability due to inconsistent antibody performance (57). However, advances in recombinant antibody production including the use of highly diverse phage display and antibody library technologies should help overcome this bottleneck (58, 59).

We have specifically demonstrated that FXYD3 and CEACAM5 are plasma membrane-bound antigens expressed preferentially in PrAd and NEPC, respectively, based on multilevel validation studies on prostate cancer cell lines and tissues. We evaluated the normal tissue expression of these antigens as an additional filter to determine the potential for off-tumor, on-target toxicities of antibodies or cell-based immunotherapies. Due to the clinical need for novel therapies for aggressive NEPC, combined with our characterization of CEACAM5 expression in NEPC (including small-cell prostate cancer) and normal tissues, we have engineered CEACAM5 CAR constructs and demonstrated their potent antigen-specific NEPC cytotoxicity. CEACAM5 is

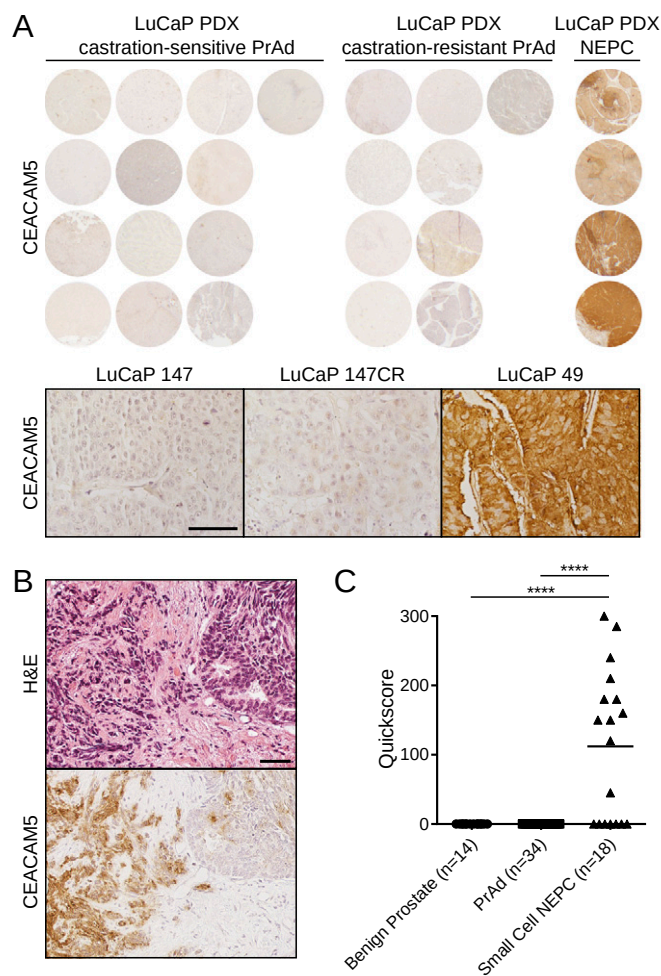


Fig. 6. CEACAM5 is a prostate cancer cell-surface antigen specific to the NEPC subtype. (A) CEACAM5 IHC of a LuCaP PDX tissue microarray with androgen-sensitive PrAd samples ($n = 13$), castration-resistant PrAd samples ($n = 9$), and NEPC samples ($n = 4$). CEACAM5 immunohistochemical stains of representative androgen-sensitive PrAd (LuCaP 147), castration-resistant PrAd (LuCaP 147CR), and NEPC (LuCaP 49) sections. (Scale bar, 100 μm .) (B) H&E and CEACAM5 immunohistochemical stains of a small cell NEPC sample archived at UCLA demonstrating adjoining regions of small-cell NEPC (left) and PrAd (right). (Scale bar, 100 μm .) (C) Quantitation of CEACAM5 IHC in benign prostate tissues ($n = 14$), PrAd ($n = 34$), and small-cell NEPC samples ($n = 18$) by Quickscore (intensity $\times$ percentage of positive cells; maximum score is 300). **** $P < 0.0001$ (by one-way ANOVA statistical analysis).

an attractive therapeutic target in several solid tumors, but the translation of CEACAM5-targeted therapies is overwhelmingly focused on advanced colorectal cancer. A number of immune-based strategies have shown preclinical efficacy and are under clinical investigation, including antibody–drug conjugates (46, 60), a CEACAM5 and CD3 bispecific antibody (61), adoptive TCR transfer (48), and CAR T immunotherapy (47).

Our preliminary results indicate that CEACAM5-directed CAR T immunotherapy warrants further investigation as a treatment strategy for NEPC. Future studies will need to assess the antitumor efficacy and potential for toxicity in the gastrointestinal tract (48) in relevant, immune-competent model systems. Additionally, strategies to enhance the specificity and alleviate potential off-tumor toxicity of CEACAM5 CARs in NEPC should also be explored. One such approach is to use dual-gate CARs (62), in which two CARs, one targeting CEACAM5 and the other a second tumor antigen with nonoverlapping expression with CEACAM5 in normal tissues, are coexpressed in T cells

such that each individual CAR is insufficient to induce a T cell response, but when both CARs are engaged, they synergize to promote T cell activation. Lastly, CARs targeting PSCA or PSMA and CEACAM5 either together or as a single bispecific construct (63) should be evaluated for safety and efficacy as a strategy to address the heterogeneity of advanced CRPC.

Methods

Detailed descriptions of cell lines, mouse xenograft studies, prostate cancer tissue microarrays and sections, antibodies, IHC, flow cytometry, and lentiviral vectors are found in [SI Appendix, SI Methods](#). Viable human cells and tissues were provided in a deidentified manner and were therefore exempt from Institutional Review Board approval. All animal studies were performed according to protocols approved by the Animal Research Committee at University of California, Los Angeles.

Bioinformatic Derivation of Genes Encoding the Cell Surfaceome. Genes encoding cell-surface proteins were assembled based on Gene Ontology annotations (22) (Membrane, Plasma Membrane, Integral Components of the Membrane, and Integral Components of the Plasma Membrane), putative transmembrane proteins based on analysis of the UniProt proteome of *Homo sapiens* using TMHMM (Version 2.0) (23), and predictions of GPI-anchored proteins from PredGPI (64).

RNA-Seq. RNA was isolated from human prostate cancer cell lines by using an miRNeasy Mini Kit (Qiagen). Libraries for RNA-seq were prepared by using a TruSeq RNA Library Prep Kit (Version 2; Illumina). Sequencing was performed on an Illumina HiSeq 3000 with 2×150 -bp reads. Demultiplexing of reads was performed by using CASAVA software (Version 1.8.2; Illumina). The Toil RNA-Seq Pipeline developed by the Computational Genomics Laboratory at the Genomics Institute of the University of California, Santa Cruz, was run locally to obtain gene- and transcript-level RSEM quantification of expression (65).

Transcriptome Analysis. FASTQ files from the Beltran 2016 RNA-Seq dataset were downloaded from dbGaP (study accession no. phs000909.v1.p1) and analyzed with the Toil RNA-Seq Pipeline. The TCGA and NIH GTEx Toil RNAseq Recompute datasets were downloaded from the University of California, Santa Cruz, Xena Public Data Hub (65). In each prostate cancer gene expression dataset analyzed, differentially expressed cell-surface genes between NEPC and PrAd samples [false discovery rate (FDR) < 0.05] were ranked based on the magnitude of fold change. RRHO analysis was performed in pairwise comparisons of gene-expression datasets as described (25). For PANTHER analysis, cell-surface genes enriched more than eightfold in either NEPC or PrAd samples in the Beltran 2016 dataset were submitted for overrepresentation testing as described (27). Rank overlap analysis was performed by taking the 500 most differentially enriched cell-surface genes in NEPC and PrAd samples from each dataset (FDR < 0.05) and identifying genes similarly enriched across all datasets.

Proteomic Analysis. A total of 4×10^7 cells from each cell line were subjected to cell-surface biotinylation and quenching per the Pierce Cell Surface Protein Isolation Kit (Thermo Fisher Scientific). Cells were lysed in urea lysis buffer (8 M urea, 2% SDS, and 100 mM Tris, pH 8) and DNA digested with 250 U of Benzonase endonuclease (Sigma). Biotin-labeled proteins were affinity-purified on streptavidin agarose beads (Thermo Fisher Scientific), sequentially treated with 5 mM Tris(2-carboxyethyl) phosphine and 10 mM iodoacetamide, and digested on-bead with Lys-C and trypsin proteases as described (66). Peptides were fractionated by multidimensional chromatography followed by tandem mass spectrometric analysis on a LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific). RAW-Xtract (Version 1.8) was used to extract peak list information from Xcalibur-generated RAW files. Database searching of the MS/MS spectra was performed by using the ProLuCID algorithm (Version 1.0). Other database search parameters included (i) precursor ion mass tolerance of ± 20 ppm; (ii) fragment ion mass tolerance of ± 400 ppm; (iii) only peptides with fully tryptic ends were considered candidate peptides in the search with no consideration for missed cleavages; and (iv) static modification of +57.02156 on cysteine residues. Peptide identifications were organized and filtered by using the DTASelect algorithm, which uses a linear discriminant analysis to identify peptide-scoring thresholds that yield a peptide-level FDR of $< 5\%$ as estimated by using a decoy database approach. Proteins were considered present in the analysis if they were identified by two or more peptides using the 5% peptide-level FDR.

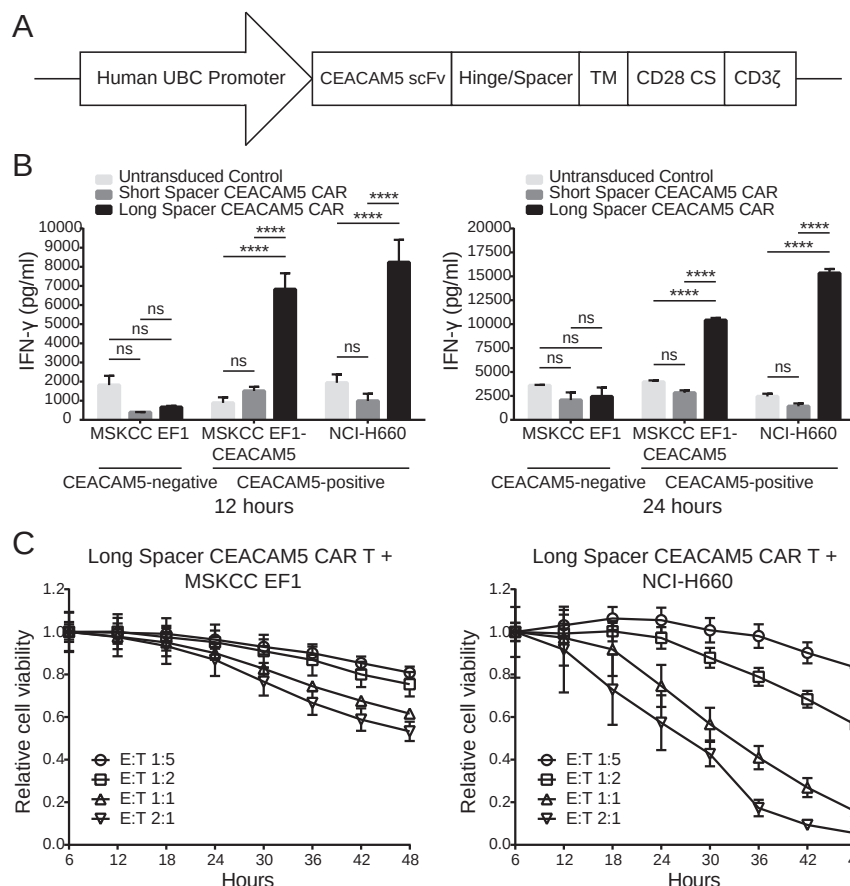


Fig. 7. Targeting CEACAM5 in NEPC with CAR T cell immunotherapy. (A) Schematic of the CAR construct targeting CEACAM5. CS, costimulatory domain; TM, CD28 transmembrane domain. (B) IFN- γ quantitation in the medium at 12 and 24 h after coculture of short spacer CEACAM5 CAR-transduced, long spacer CEACAM5 CAR-transduced, or untransduced T cells with CEACAM5-negative or -positive target cell lines as shown. SE measurements for four replicate wells are displayed. Data are representative of three independent experiments with similar results. ns represents nonsignificance. **** $P < 0.0001$ (by two-way ANOVA statistical analysis). (C) Relative viability over time of CEACAM5-negative MSKCC EF1 target cells or CEACAM5-positive NCI-H660 target cells cocultured with long spacer CEACAM5 CAR-transduced T cells. Effector-to-target ratios varying from 1:5 to 2:1 are shown. SE measurements for three replicate wells at each timepoint are displayed. Data are representative of two independent experiments with similar results.

CAR T cell Engineering and Coculture Assays. Deidentified human PBMCs were obtained from the UCLA Virology Core Laboratory and grown in T cell medium (TCM) base medium composed of AIM V medium (Thermo Fisher Scientific), 5% heat-inactivated human AB serum, 2 mM glutamine, and 55 μ M 2-mercaptoethanol (Sigma). For coculture experiments involving IFN- γ release assays measured by ELISA, human PBMCs were activated in a 24-well plate coated with 1 μ g/mL anti-CD3 (eBioscience OKT-3), 1 μ g/mL anti-CD28 (eBioscience CD28.2), and 300 U/mL IL-2 in TCM base medium. After 48 h, cells were spin-infected daily for 2 d with CAR lentivirus at a multiplicity of infection (MOI) of ~ 6 –11 in TCM base medium, 300 U/mL IL-2, and 8 μ g/mL polybrene. After each infection, the cells were washed and grown in TCM base medium with 300 U/mL IL-2. Ninety-six hours after final spin infection, T cell transduction efficiency was measured by flow cytometry, and T cells were cocultured with target cells at a target:effector ratio of 1:1. Supernatant was harvested at 12 and 24 h after coculture. IFN- γ was quantitated with the BD OptEIA Human IFN- γ ELISA Set (BD Biosciences) according to the manufacturer's protocol. For coculture experiments with direct visualization of cytotoxicity by live cell imaging, human PBMCs were activated with Gibco Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific) in TCM base medium with 50 U/mL IL-2 at a cell:bead ratio of 1. After 96 h, T cells were infected with CAR lentivirus by spin infection in TCM base medium with 50 U/mL IL-2 at an MOI of 0.5–50. Cells were washed 24 h after infection and cultured in TCM base medium with 50 U/mL IL-2. Dynabeads were removed 48 h after infection. Ninety-six hours after spin infection, T cell transduction

efficiency was measured by flow cytometry, and T cells were cocultured with target cells at a range of target:effector ratios. Cytotoxicity was measured by Incucyte ZOOM through quantification of GFP-positive target cell counts.

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Identification of Cell Surface Targets for CAR T Cell Immunotherapy

Diana C. DeLucia and John K. Lee

Abstract

Immunotherapy has become a prominent approach for the treatment of cancer. Targeted killing of malignant cells by adoptive transfer of chimeric antigen receptor (CAR) T cells is a promising immunotherapy technique in oncology. However, the identification of cell surface antigens unique to tumor cells against which CAR T cells can be engineered has historically been challenging and not well documented in solid tumors. Here, we describe a generalized method to construct a cell subtype-specific surface antigen profile (i.e., surfaceome) from cell lines and identify high-confidence antigens as effective targets for CAR T cell therapy by integrating transcriptomics and cell surface proteomics. This method is widely applicable to all therapies utilizing CAR T cells, such as cancer, as well as infectious and autoimmune diseases.

Key words Cell surface antigens, Chimeric antigen receptor, CAR T cell, RNA sequencing, Transcriptomics, Proteomics, Mass spectrometry

1 Introduction

Chimeric antigen receptor (CAR) T cells are T lymphocytes engineered to express a unique receptor that recognizes a specific cell surface antigen and mediates cytotoxic elimination of such cells. Typically, T cells recognize antigens in the form of peptides presented on the surface of target cells in association with the major histocompatibility complex (MHC). Usually, MHC molecules are responsible for recognizing nonself or foreign antigens, such as those derived from viral or bacterial proteins, but can also recognize and bind self-antigens [1]. The interaction of the T cell receptor (TCR) on T cells with the MHC on target cells during antigen presentation is essential for the induction of T cell effector functions, such as target cell killing. CARs are unique compared to endogenous TCRs in that they contain an extracellular single-chain variable antibody fragment linked to a single TCR transmembrane domain and intracellular signaling domain [2]. The variable region

of the CAR antibody fragment is designed to recognize antigens in the form of intact protein on the surface of target cells in an MHC-independent manner. This allows CAR T cells to perform targeted killing of cells with downregulated MHC surface expression, a mechanism of immune escape observed in several tumors [3, 4] and utilized by many pathogens, such as HIV-1 [5, 6]. CAR T cell immunotherapy has had promising antitumor effects against hematological and solid tumors in preclinical studies, and numerous constructs have been assessed in clinical trials [7, 8]. Unlike the foreign pathogen-specific proteins targeted by CAR T cells in infectious disease studies, the majority of CAR T cell targets against tumors are self-antigens that are inherently expressed on various cell lineages and tissues throughout the body, which can be tolerated or cause deleterious off-target side effects [9]. To increase the likelihood of efficient tumor-specific cytotoxicity and minimal damage to normal tissue, CAR T cell antigens should ideally be high-density neoantigens or self-antigens that are exclusively expressed on tumor cells.

Historically, the comparison of healthy and malignant cell transcriptomes has been the primary method of identifying CAR protein targets against cancer. This method relies on the assumption that mRNA expression equates to and properly represents protein expression on the cell surface. However, the quantitative relationship between mRNA and surface protein expression is often unpredictable due to the number of variables affecting surface expression of proteins following gene transcription, including mRNA translation, and posttranslational protein regulation and trafficking [10]. Here, we explain an integrated method to identify high-confidence protein antigens for CAR T cell therapy using transcriptomic and proteomic data [11]. The method is broken into three parts: (1) RNA sequencing and differential gene expression analysis, (2) generation of cell-specific surface protein profiles, and (3) integrated cell surface transcriptome and proteome analysis. Importantly, this is a generalized method that can be applied to CAR antigen identification in the context of any available cell type or disease of interest for which the appropriate proteomic and transcriptomic datasets are available.

2 Materials

2.1 RNA Extraction and Library Preparation

1. miRNeasy Mini Kit (Qiagen).
2. Chloroform.
3. Diethyl pyrocarbonate (DEPC)-treated water. Add 0.1% volume/volume DEPC to distilled water. Mix well, and incubate at 37 °C for 8–12 h. Autoclave for at least 15 min to inactivate DEPC. Alternatively, DEPC-treated water may be purchased.
4. TruSeq RNA Library Prep Kit v2 (Illumina).

2.2 Cell Surface Protein Biotinylation and Cell Lysis

1. Pierce Cell Surface Protein Isolation Kit (Thermo Fisher Scientific).
2. Cell scrapers.
3. Lysis buffer: 8 M urea, 2% SDS, 100 mM Tris-HCl, pH 8.0. Weigh 24 g of high-purity urea and 1 g of SDS, and transfer to a 50 ml polypropylene conical centrifuge tube (*see Note 1*). Add 5 ml of 1 M Tris-HCl and molecular biology-grade water to a total volume of 50 ml. Add one tablet of the cOmplete Protease Inhibitor Cocktail (Roche). Mix on a tube revolver for at least 4 h, and ensure that the urea is completely dissolved. Adjust pH with HCl to 8.0 (*see Note 2*). Store at -20°C until use.
4. Protein LoBind conical bottom polypropylene 2.0 ml centrifuge tubes (Eppendorf).
5. Benzonase Nuclease (Sigma). Store at -20°C , and avoid repeated freeze thaw cycles.
6. Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

2.3 Immunoaffinity Purification of Cell Surface Proteins

1. Pierce High-Capacity Streptavidin Agarose (Thermo Fisher Scientific).
2. Reductive Alkylation Kit from Hampton Research (*see Note 3*).
3. GELoader 0.5–20 μl tips from Eppendorf.
4. Lysis buffer, as above.
5. 1 M Dimethylamine borane complex.
6. 1 M Methanol-free formaldehyde.
7. Wash buffer A: 8 M urea, 2% SDS, 100 mM Tris-HCl, 500 mM NaCl, pH 8.0. Weigh 24 g of urea and 1 g of SDS, and transfer to a 50 ml polypropylene conical centrifuge tube. Add 5 ml of 1 M Tris-HCl, 5 ml of 5 M NaCl, and molecular biology-grade water to a total volume of 50 ml. Mix on a tube revolver for at least 4 h, and ensure that the solutes are completely dissolved. Adjust pH with HCl to 8.0. Store at -20°C until use.
8. Wash buffer B: 8 M urea, 0.2% SDS, 100 mM Tris-HCl, 500 mM NaCl, 5% ethanol, 5% isopropanol, pH 8.0. Weigh 24 g of urea and 100 mg of SDS, and transfer to a 50 ml polypropylene conical centrifuge tube. Add 5 ml of 1 M Tris-HCl, 5 ml of 5 M NaCl, 2.5 ml absolute ethanol, 2.5 ml isopropanol, and molecular biology-grade water to a total volume of 50 ml. Mix on a tube revolver for at least 4 h, and ensure that the solutes are completely dissolved. Adjust pH with HCl to 8.0. Store at -20°C until use.
9. Digestion buffer: 8 M urea, 100 mM Tris-HCl, pH 8.5. Weigh 24 g of high-purity urea and 100 mg of SDS, and transfer to a 50 ml polypropylene conical centrifuge tube. Add 5 ml of 1 M Tris-HCl and molecular biology-grade water to a total volume of 50 ml (*see Note 4*). Mix on a tube revolver for at

least 4 h, and ensure that the solutes have completely dissolved. Adjust pH with HCl to 8.5. Store at -20°C until use.

2.4 Tryptic Digestion of Purified Proteins

1. 200 mM Tris(carboxyethyl)phosphine (TCEP). Add 2.293 g of TCEP to a 50 ml polypropylene conical centrifuge tube. Add 35 ml of cold molecular biology-grade water to the tube, and dissolve the TCEP (*see Note 5*). Adjust pH with NaOH to 7.0. Add molecular biology-grade water to a final volume of 40 ml. Aliquot and store at -20°C .
2. 500 mM Iodoacetamide. Add 0.046 g iodoacetamide to 500 μl of water (*see Note 6*).
3. 0.1 $\mu\text{g}/\mu\text{l}$ Lys-C. Reconstitute 5 μg lyophilized, sequencing-grade Lys-C in 50 μl molecular biology-grade water. Aliquot and store at -20°C .
4. 100 mM Tris-HCl, pH 8.5. Prepare solution using high-purity, sterile 1 M Tris-HCl, pH 8.5, and molecular biology-grade water.
5. 100 mM CaCl_2 . Dissolve 1.47 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 100 ml of molecular biology-grade water. Aliquot and store at 4°C .
6. 0.4 $\mu\text{g}/\mu\text{l}$ Trypsin. Reconstitute 20 μg lyophilized, mass spectrometry-grade trypsin in 50 μl 50 mM acetic acid. Aliquot and store at -80°C .
7. 98% Formic acid.

3 Methods

3.1 High-Throughput RNA Sequencing (RNA-Seq)

1. Expand cell lines of interest in 100 mm tissue culture dishes.
2. Follow the miRNeasy Mini Kit protocol (Qiagen) to isolate RNA from the cell lines (*see Note 7*). Elute RNA in RNase-free DEPC-treated water.
3. Submit aliquots of cell line RNA samples for analysis on a TapeStation Automated Electrophoretic System (*see Note 8*).
4. Store RNA samples at -80°C .
5. Perform library preparation according to the TruSeq RNA Library Prep Kit v2 protocol (Illumina) (*see Note 9*).
6. Submit libraries for high-throughput sequencing on an Illumina sequencing system (*see Note 10*).

3.2 Differential Expression Analysis of Genes Encoding Putative Cell Surface Proteins

1. Generate a set of genes encoding cell surface proteins. The human cell surfaceome characterized by mass spectrometry is described in multiple manuscripts and publicly available databases including the Cell Surface Protein Atlas [12].
2. After mapping reads and calculating gene-level counts, perform differential gene expression analysis between cell line

sample classes based on an appropriate statistical cutoff (*see Note 11*).

3. From the output of differentially expressed genes, extract and sort genes encoding cell surface proteins identified in **step 1** based on decreasing log-fold change.

3.3 Cell Surface Protein Isolation

1. Expand cell lines of interest in T75 cm² vented cap tissue culture flasks. Determine the cell count when 90–95% confluent.
2. Grow cell lines in a sufficient number of T75 cm² tissue culture flasks for a goal of 4×10^7 cells (*see Note 12*).
3. Follow the Pierce Cell Surface Protein Isolation Kit protocol through Part A: Biotinylation to perform biotinylation of cell surface proteins, quenching of the reactions, and cell washing.
4. Perform cell lysis by adding 2 ml of lysis buffer with protease inhibitor cocktail to each cell pellet, and transfer each lysate to an Eppendorf Protein LoBind 2.0 ml centrifuge tube.
5. Add 1 μ l Benzonase Nuclease to each sample, and place on a microcentrifuge rotator at room temperature for 30 min (*see Note 13*).
6. Centrifuge the samples at $20,000 \times g$ for 15 min at room temperature.
7. Collect the supernatants using a 1 ml micropipette, avoiding the lipid layer on top of the supernatant, and transfer to new Eppendorf Protein LoBind 2.0 ml centrifuge tubes.
8. Perform total protein quantitation of each sample using the Pierce BCA Protein Assay Kit.
9. Normalize the concentration of the protein lysates by adding appropriate volumes of lysis buffer to the samples (*see Note 14*).

3.4 Affinity Purification and Digestion of Isolated Proteins for Mass Spectrometry

1. Transfer 1 ml of Pierce High-Capacity Streptavidin Agarose into a 1.5 ml centrifuge tube. Pellet agarose beads in a microcentrifuge at $100 \times g$ for 2 min.
2. Remove supernatant without removing agarose beads. Add 1 ml of ice-cold phosphate-buffered saline (PBS). Pellet agarose beads in a microcentrifuge at $100 \times g$ for 2 min. Repeat a total of five times to equilibrate the agarose beads.
3. After the final spin, add 1 ml of ice-cold PBS, and proceed to reductive methylation of the streptavidin agarose beads below in **steps 4** through **7**.
4. Add 20 μ l 1 M dimethylamine borane complex and 40 μ l 1 M methanol-free formaldehyde to the microcentrifuge tube (*see Note 15*). Rotate at 4 °C for 2 h.

5. Add an additional 20 μ l 1 M dimethylamine borane complex and 40 μ l 1 M methanol-free formaldehyde to the microcentrifuge tube. Rotate at 4 °C for 2 h.
6. Add an additional 10 μ l 1 M dimethylamine borane complex to the microcentrifuge tube. Rotate at 4 °C overnight.
7. Add 125 μ l 1 M glycine, pH 8.6, and 125 μ l 50 mM DTT to the microcentrifuge tube. Rotate at 4 °C for 2 h.
8. Wash the reductively methylated streptavidin agarose beads by adding 1 ml of ice-cold PBS, pelleting the agarose beads in a microcentrifuge at $100 \times g$ for 2 min, and removing the supernatant without removing agarose beads. Repeat a total of ten times.
9. Add 400 μ l of ice-cold PBS, and store at 4 °C.
10. Transfer 125 μ l of reductively methylated streptavidin agarose beads to an Eppendorf Protein LoBind 2.0 ml centrifuge tube for each sample.
11. Add 1 ml lysis buffer to each tube, rotate tubes for 3 min at room temperature, pellet the agarose beads in a microcentrifuge at $100 \times g$ for 2 min, and remove supernatant. Repeat a total of three times to equilibrate the agarose beads.
12. Load 2 ml of lysates normalized for total protein concentration onto the agarose beads. Rotate tubes for 2 h at room temperature.
13. Pellet the agarose beads in a microcentrifuge at $100 \times g$ for 2 min, and remove supernatant.
14. Add 1 ml of wash buffer A, rotate the tube for 5 min at room temperature, pellet the agarose beads in a microcentrifuge at $100 \times g$ for 2 min, and remove supernatant. Repeat two more times.
15. Add 1 ml of wash buffer B, rotate the tube for 5 min at room temperature, pellet the agarose beads in a microcentrifuge at $100 \times g$ for 2 min, and remove supernatant. Repeat two more times.
16. Add 1 ml of digestion buffer, rotate the tube for 5 min at room temperature, pellet the agarose beads in a microcentrifuge at $100 \times g$ for 2 min, and remove supernatant. Repeat two more times.
17. Add 1 ml digestion buffer to the agarose beads, and transfer to new Eppendorf Protein LoBind 2.0 ml centrifuge tubes.
18. Perform three washes with digestion buffer as in **step 16**.
19. Pellet the agarose beads in a microcentrifuge at $100 \times g$ for 5 min. Carefully aspirate as much liquid as possible using Eppendorf GELoader tips without removing agarose beads.
20. Resuspend the agarose beads in 50 μ l digestion buffer (*see Note 16*).

21. Add 1.25 μl of 200 mM TCEP to each tube. Shake at 1300 rpm at room temperature for 20 min.
22. Add 1.2 μl of 500 mM iodoacetamide to each tube. Shake in the dark at 1300 rpm at room temperature for 20 min.
23. Add 2.5 μl of 0.1 $\mu\text{g}/\mu\text{l}$ Lys-C to each tube. Shake in the dark at 1300 rpm at 37 °C for 4 h.
24. Add 2 μl of 100 mM CaCl_2 to each tube.
25. Add 4 μl of 0.4 $\mu\text{g}/\mu\text{l}$ trypsin. Shake in the dark at 1300 rpm at 37 °C for 16 h.
26. Add 10.33 μl of 98% formic acid. Shake in the dark at 1300 rpm at room temperature for 5 min.
27. Spin tubes at $20,800 \times g$ at room temperature for 10 min. Collect and transfer the supernatants to new Eppendorf Protein LoBind 1.5 ml centrifuge tubes (*see* **Note 17**).
28. Repeat **step 27**.
29. Split supernatant into two new Eppendorf Protein LoBind 1.5 ml centrifuge tubes. Store at -80°C .
30. Submit samples to your local proteomics core facility for desalting and tandem mass spectrometry.

3.5 Integrative Cell Surface Transcriptome and Proteome Analysis

1. Use protein quantitation data analyzed from the mass spectrometry data to perform differential expression analysis on identified proteins between cell line classes based on an appropriate statistical cutoff (*see* **Note 18**).
2. From the output of differentially expressed proteins, extract and sort only cell surface proteins from Subheading 3.2, **step 1** based on decreasing log-fold change.
3. Perform Rank-Rank Hypergeometric Overlap analysis [13] as described in the user guide using the online tool (<http://systems.crupp.ucla.edu/rankrank/rankranksimple.php>) with ranked differentially expressed genes (rank 1) and ranked differentially expressed proteins (rank 2) between cell line classes (*see* **Note 19**). Examine the output of genes/proteins within regions of maximal statistical overlap for high-confidence cell line class-specific cell surface proteins.

4 Notes

1. Wear a mask and gloves when measuring SDS.
2. Concentrated HCl (10 N) may be used initially followed by HCl with lower ionic strength (5 N and 1 N) to avoid overshooting the target pH.
3. Reagents found in the Reductive Alkylation Kit may be purchased individually at reduced cost.

4. Use high-purity, sterile 1 M Tris-HCl and pH 8.5 which can be purchased from Alfa Aesar or Teknova.
5. When TCEP is dissolved in water, the solution is highly acidic with a pH of about 2.5.
6. Use HPLC-grade iodoacetamide. Always prepare this solution fresh.
7. Collect cell lines when equally 90–95% confluent. Cell lines lysed in Qiazol Lysis Reagent may be stored at -80°C for several weeks without significant RNA degradation.
8. The optimal RNA integrity number (RIN) from TapeStation analysis should be >8 to proceed with library preparation and high-throughput RNA sequencing. If the RIN is <8 , recommend repeating the extraction of RNA from cell line samples in culture.
9. Ensure that each sample is prepared with a unique indexed adapter to enable multiplexing of multiple libraries in lanes of the flow cell.
10. Generally, we have used an Illumina HiSeq 3000 system with paired-end 150 bp reads and four libraries multiplexed on each lane of the flow cell. Using this approach, we obtain between 60 and 80 million mapped reads per sample. However, single-end short read length protocols are usually sufficient for gene expression analysis.
11. We have used the Toil RNA-seq Pipeline [14] to perform RNA-seq expression quantification. Rounded, estimated RSEM gene-level counts are analyzed in DESeq2 [15] for differential gene expression analysis. Typically, we use a q -value cutoff of ≤ 0.05 .
12. Approximately 4–6 T75 cm^2 tissue culture flasks with cells grown to 90–95% confluence will yield 4×10^7 cells.
13. Benzonase Nuclease is added to enzymatically degrade nucleic acids from the sample. The viscosity of the cell lysate should be significantly reduced after this step. If not, repeat this step with fresh Benzonase Nuclease. Alternatively, a handheld sonicator can be used at low power to disrupt the cells on ice using multiple bursts of 1 s each.
14. Normalized protein lysates may be stored at -80°C for several weeks.
15. We recommend using Parafilm to seal the cap of the microcentrifuge tube to prevent any leakage of contents while on the rotator.
16. Consult with your local proteomic core facilities as most will accept protein samples and perform or assist with on-bead digestion followed by desalting for mass spectrometry.

17. Avoid transferring agarose beads with the supernatant.
18. We have used log-transformed normalized abundance (NSAF₅) to perform differential protein analysis between cell line classes.
19. The tab-delimited text input for Rank-Rank Hypergeometric Overlap analysis is stringent. Carefully follow the formatting instructions. Each row representing a specific gene/protein must include both a differential gene rank (rank 1) and a differential protein rank (rank 2). If either of these values is missing due to not meeting statistical criteria, the gene/protein should be removed from the analysis.

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Identification of Therapeutic Vulnerabilities in Small-cell Neuroendocrine Prostate Cancer

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ABSTRACT

Purpose: Small-cell neuroendocrine prostate cancer (SCNPC) exhibits an aggressive clinical course and incidence rates seem to be increasing following resistance to potent androgen receptor (AR) antagonists. Currently, treatment options are limited and few model systems are available to identify new approaches for treatment. We sought to evaluate commonalities between SCNPC and other aggressive neuroendocrine carcinomas to identify therapeutic targets.

Experimental Design: We generated whole transcriptome RNA-sequencing data from AR-active prostate cancers (ARPCs) and SCNPCs from tumors collected at rapid autopsy and two other neuroendocrine carcinomas, Merkel cell carcinoma (MCC), and small-cell lung cancer. We performed cross-tumor comparisons to identify conserved patterns of expression of druggable targets. We tested inhibitors to highly upregulated drug targets in a panel

of prostate cancer cell lines and *in vivo* patient-derived xenograft (PDX) models.

Results: We identified BCL2 as highly upregulated in SCNPC compared with ARPC. Inhibitors targeting BCL2 induced apoptotic cell death in SCNPC cell lines at nanomolar concentrations while ARPC cell lines were resistant. Treatment with the BCL2 inhibitor navitoclax leads to a reduction of growth of SCNPC PDX tumors *in vivo*, whereas ARPC PDX models were more resistant. We identified Wee1 as a second druggable target upregulated in SCNPC. Treatment with the combination of navitoclax and the Wee1 inhibitor AZD-1775 repressed the growth of SCNPC PDX resistant to single-agent BCL2 inhibitors.

Conclusions: The combination of BCL2 and Wee1 inhibition presents a novel therapeutic strategy for the treatment of SCNPC.

Introduction

Prostate cancer is notable for a general dependency on androgen receptor (AR) signaling for survival and proliferation, a feature that has served as the major focal point of treating metastatic prostate cancer for decades. However, a small subset of prostate cancers exhibits histologic characteristics of small-cell carcinomas similar to small-cell cancers found in other organs with small blue-round-cell morphology and the expression of proteins associated with neuroendocrine functions such as chromogranin and synaptophysin (1). These carcinomas lack AR activity and respond poorly to AR-directed therapy. Although *de novo* small-cell prostate cancer is very rare representing <1% of all prostate cancers at diagnosis (2), in the context of intense therapeutic pressures designed to antagonize AR signaling, a subset of resistant prostate cancers lose or attenuate the expression of AR-regulated genes and gain

the expression of neuroendocrine-associated genes without exhibiting small-cell morphology. These prostate cancers that we collectively refer to as small cell or neuroendocrine prostate cancer (SCNPC) respond poorly to conventional therapies and have substantially worse outcomes. Overt small-cell prostate cancers and the treatment-associated SCNPCs frequently demonstrate biallelic inactivation of *RB1* and *TP53* tumor suppressor genes. Of interest, treatment-associated SCNPC often carry genomic alterations identical to the original adenocarcinoma, such as the *TMPRSS2-ERG* gene rearrangement, indicating either a common progenitor or that SCNPC arises from the transdifferentiation of an existing adenocarcinoma following repression of the major lineage-directing AR program. Transdifferentiation has been described in other malignancies such as lung adenocarcinoma where resistance to EGFR inhibition is associated with the emergence of small-cell lung cancer (SCLC) phenotypes (3, 4). The molecular events regulating transdifferentiation and the acquisition of neuroendocrine characteristics are active areas of investigation.

The aggressive behavior of SCNPC underscores a critical need in the field to identify effective therapeutic strategies. One approach, centered on defining the major differences in oncogenic drivers between AR-active prostate cancers and those with SCNPC characteristics, identified the *MYCN* proto-oncogene, bHLH transcription factor (*MYCN*) (5, 6), an oncogene previously implicated in the initiation of other neuroendocrine tumor types such as neuroblastoma and whose expression and/or amplification is also associated with SCLC (7–9). A common mechanism has been proposed by which Aurora kinase A stabilizes N-Myc protein in both *MYCN*-amplified neuroblastoma and *MYCN*-amplified SCNPC (5, 10). Clinical trials with the Aurora kinase inhibitor alisertib have demonstrated that a subset of patients with molecular features supporting Aurora-A and N-Myc activation exhibited significant clinical benefit (11).

To date, most studies of SCNPC have focused on comparing tumors of prostate origin. However, the rarity of SCNPC and lack of model

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Translational Relevance

BCL2 inhibition may have single-agent efficacy in subsets of SCNPC. The efficacy of the dual inhibition of BCL2 and Wee1 in preclinical models represents a therapeutic strategy with the potential to overcome resistance to BCL2 inhibitors.

systems with which to interrogate the disease and test potential therapeutics has constrained progress. Recent “pan-cancer” analyses indicate that comparisons of cancer subtypes arising in divergent organ sites can yield information with respect to cell of origin, common developmental processes, and actionable targets. Notably, a pan-cancer analysis identified an adult stem cell signature that associates with aggressive variants across epithelial neoplasms and which was particularly elevated in small-cell/neuroendocrine tumors (12).

In this study, our objective was to identify therapeutic vulnerabilities in SCNPCs. To ascertain potential targets, we evaluated the transcriptional programs of three tumor types that exhibit small-cell and/or neuroendocrine features: SCNPC, SCLC, and Merkel cell carcinoma (MCC). Focusing on the druggable genome, we identified several candidates including BCL2 and Wee1 and demonstrated preclinical *in vitro* and *in vivo* efficacy of clinical inhibitors against BCL2 and Wee1. These studies provide rationale to evaluate the efficacy of BCL2- and Wee1-directed therapy in patients with SCNPC.

Materials and Methods

Patient sample and RNA collection

Prostate cancer metastases were collected as part of the Prostate Cancer Donor Program at the University of Washington (UW; Seattle, WA). Collection of samples and RNA isolation of UW prostate cancer samples were carried out as previously described (13). Merkel cell carcinoma patient samples and RNA were collected as part of a previously published study (14). Samples of patients with SCLC were obtained through the Cooperative Human Tissue Network. The SCLC cell lines NCI-H1436, NCI-H1672, NCI-H1963, NCI-H2141, NCI-H2195, NCI-H735, and NCI-H774 were obtained from ATCC. RNA from tumor samples and cell lines was extracted with TRIzol (Invitrogen).

RNA-sequencing library prep and read processing

The purity and concentration of RNA was assessed by Nanodrop (Thermo Fisher Scientific) and Agilent Bioanalyzer. One microgram of total RNA was used as input to either the Illumina Tru Seq RNA Library Kit v2 or the Illumina TruSeq Stranded mRNA Library prep kit and libraries were prepared and bar coded according to the manufacturer's protocol. Libraries were sequenced on the Illumina HiSeq 2500 generating either 50 or 75 base pair paired end reads. Resulting reads were mapped to the hg38 human genome with TopHat v2.0.14 and transcript abundance was measured using the R Bioconductor package Genomic Alignments v1.18.0. Sequencing reads from patient-derived xenograft (PDX) libraries were aligned to both hg38 human and mm10 mouse genomes.

Statistical analyses

Differential expression analyses of RNA-sequencing data were carried out using the Bioconductor package edgeR v3.24.2 with transcript abundances as input. An FDR threshold of <0.05 was used as a cutoff for differential expression assessment. Multidimensional

scaling analysis was conducted with all measured genes as input to the R Bioconductor package limma v3.38.3.

Calculation of AR and neuroendocrine signature scores was carried out using the Bioconductor package GSVA v1.30.0 using z-score normalized log₂ fragments per kilobase of transcript per million mapped reads (FPKM) values as input. Scatterplots were created using ggplot2 v3.1.0 and the Pearson correlation between gene and signature score was calculated using the ggpubr v0.2 stat.cor() function.

Boxplots for individual genes were created with ggplot2 v3.1.0 and statistical assessment between groups was assessed by Student *t* test using the ggpubr v0.2 stat_compare_means() function.

Guilt-by-association analysis was performed by calculating the Pearson correlation between the log₂ FPKM expression of the lncRNA with that of each protein coding gene across all samples. Absolute values of the Pearson correlations were sorted and the top 1% most highly correlated protein coding genes was used as input to identify enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with the gProfiler R package. Additional KEGG enrichment analysis was also performed with the gProfiler R package.

RNA-seq data from previously published datasets Bluemn and colleagues, 2017, Aggarwal and colleagues, 2018, and Abida and colleagues 2019 were analyzed and sample phenotypic groups were assigned for each cohort using classical multidimensional scaling calculated with the cmdscale function in R on the expression profiles of 34 genes from the combined lists of “NEURO I,” “NEURO II,” and “AR” gene signatures in the study by Labrecque and colleagues (15) and the “neuroendocrine” and “AR” signatures in the study by Bluemn and colleagues (13). The distance metric was “euclidean” calculated by dist function on the columns (samples).

Cell lines

The cell lines, LNCaP (ATCC), C4-2 (ATCC), MSKCC EF1 (gift from John K. Lee), MKL-1 (Sigma), VCaP (ATCC), LAPC4 (gift of Charles Sawyers), NCI-H660 (ATCC), NCI-H82 and NCI-H69 (ATCC), and MS-1 (Sigma) were maintained in a 37°C incubator with 5% CO₂ and grown in medium supplemented with FBS and other additives as recommended by ATCC or the cell line provider. Short tandem repeat genotyping was used to authenticate the lines and cells were confirmed to be *Mycoplasma* free using the MycoAlert Detection Kit (Lonza, LT07-418). Cells were cultured no longer than 10 passages after thawing and before experimental use.

Protein isolation and immunoblotting

Cell lines were washed 1× with PBS prior to the addition of a cell lysis buffer (1.5 mol/L urea, 1% SDS, 1% NP-40, 2% Tween 20, 250 nmol/L NaCl, PBS) supplemented with 1× phosphatase inhibitors (PhosStop, Roche Diagnostics) and a 1× protease inhibitor cocktail (Complete Mini, Roche Diagnostics). Patient-derived xenograft tissues were pulverized in liquid nitrogen with a mortar and pestle prior to the addition of lysis buffer. Lysates were sonicated and then centrifuged to clear debris. Protein quantification was performed using the Pierce bicinchoninic acid assay (Thermo Fisher Scientific). Lysates were run on a 4% to 12% NuPage Bis-Tris gel and protein was transferred to a nitrocellulose membrane in Tris/CAPS buffer using a semidry transfer apparatus. Membranes were blocked and incubated with antibody solutions in 5% milk powder in 1× PBS with 0.01% Tween. Primary antibodies targeting BCL2 (Santa Cruz Biotechnology, sc-7382, 1:500), BCL-XL (Cell Signaling Technology, 2764, 1:1,000), BCLW (Cell Signaling Technology, 2724, 1:1,000), cleaved caspase-3 (Cell Signaling Technology, 9661S, 1:1,000), WEE1 (Cell Signaling Technology, 13084, 1:1,000) phospho-cdc2(Tyr15; Cell Signaling Technology,

4539, 1:1,000), AR (Abcam, ab133273, 1:2,000), PSA (Santa Cruz Biotechnology, sc7638, 1:100), and GAPDH (BioRad, hFAB Rhodamine, catalog No. 12004167, 1:5,000) were used. Secondary antibodies used were goat-anti-rabbit [Goat anti-Rabbit IgG (H + L) Secondary Antibody, HRP catalog No. 31460, 1:5,000] and goat-anti-Mouse (BioRad, StarBright Blue 700 catalog No. 12004158, 1:5,000). Blots were imaged using the ChemiDoc MP (Bio-Rad).

IHC

LuCaP tissue microarrays were constructed and stained as described previously (16). Antibodies directed toward the detection of BCL2 [EPR17509] (Abcam, catalog No. ab182858), p-CDK1 (Abcam, ab-133463), and Wee1 (Santa Cruz Biotechnology, sc-5258) were used according to the manufacturer's instructions. Staining assessment was blinded and the H-score was calculated and averaged across multiple tissue/tumor cores for the same LuCaP PDX line.

Drug treatments of cell lines

Cell lines were seeded in 96-well plates in 50- μ L media 24 hours before the addition of the inhibitor. Serial dilutions of inhibitors were made in media and 50 μ L of a 2 $\times$ inhibitor solution were added to plates containing cells. At least three technical replicates for each dose were plated. The maximal amount of DMSO for each serial dilution was used as a vehicle control for all dose points. Viability was assessed 96 hours after addition of inhibitor using the Cell Titer-Glo 2.0 Assay (Promega), following the manufacturer's protocol. Dose-response curves shown are an average and SD of three biological replicates for each cell line and each inhibitor. Nonlinear regression curves were fitted to the average of biological replicates in GraphPad Prism7. Inhibitors used were ABT-199 (Selleckchem, S8048), ABT-263 (Selleckchem, S1001), Gambogic Acid (Selleckchem, S2448), A-1155463 (Selleckchem, S7800), and AZD-1775 (Selleckchem, S1525).

For assessment of cell death with cleaved caspase-3 immunoblot, cells were plated in 10-cm dishes in media. After 24 hours, the media was changed to media containing DMSO or 100 nmol/L ABT-263. Cells were harvested 24 hours postaddition of drug and lysates collected as described.

Tumor dissociation and drug treatment

LuCaP PDX tumors were harvested, minced, and placed in digestion media containing 1 mg/mL collagenase and 1 mg/mL dispase. Then, tumor mixtures were incubated at 37°C for 1 hour. Digested tissue was passed through 20G needles and 100- μ and 40- μ filters before counting. Cells were plated in 6-well dishes and treated with vehicle (DMSO), AZD-1775 (500 nmol/L final concentration), ABT-263 (final 10 nmol/L), or the combination AZD-1775 and ABT-263 (500 nmol/L + 10 nmol/L). After 72 hours, protein was harvested.

siRNA treatment of cell lines

Cells were plated 24 hours before transfection in 6-well or 96-well plates. Cells were transfected with 60 nmol/L (final concentration) nontargeting siRNA (Dharmacon, catalog No. D-001206-13-20, siGENOME nontargeting siRNA Pool No. 1) or BCL2 siRNA (Dharmacon, catalog No. M-003307-06-0010, SMARTpool: siGENOME BCL2 siRNA) using TransIT-TKO Transfection Reagent (Mirus, catalog No. MIR 2154) following the manufacturer's instructions. Protein was collected and viability was assessed 96 hours posttransfection. Data shown are an average and SD of three biological replicates.

PDX studies

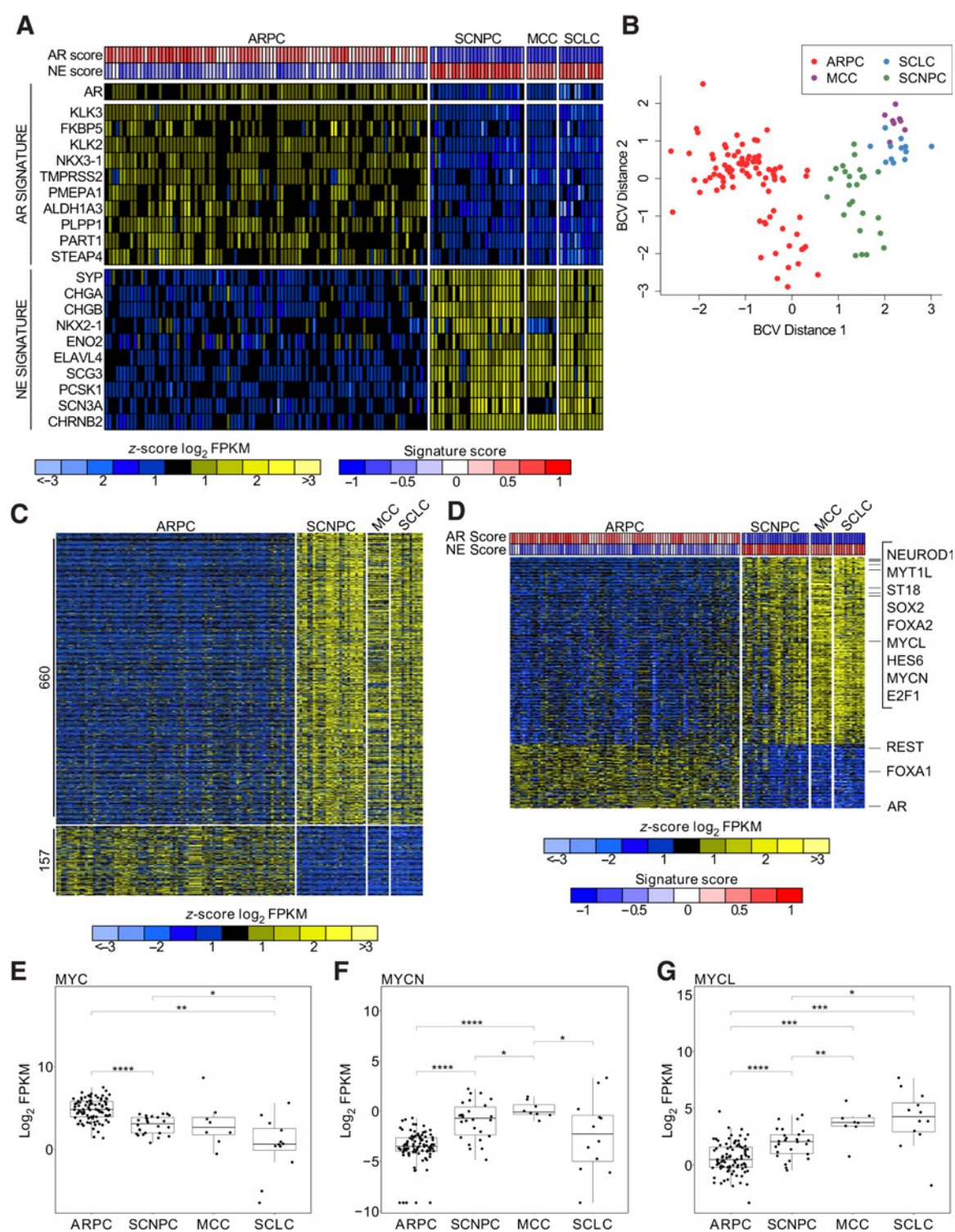
All animal procedures were approved by UW Institutional Animal Care and Use Committee and according to NIH guidelines. Male mice ($n = 24$ per PDX) were implanted subcutaneously with tumor bits using a trocar. We used LuCaP 49, LuCaP 93, LuCaP 173.1, LuCaP 145.1, and LuCaP 145.2 SCNPC PDX models. When tumors reached approximately 100 mg, animals were randomized to treatment groups. We were aiming for three animals with tumor per arm: (1) control, (2) ABT-263, (3) AZD-1775, and (4) ABT-263 + AZD-1775. ABT-263 was formulated at 50/mg/kg in 10% ethanol, 30% polyethylene glycol 400, and 60% Phosal 50PG, and AZD-1775 was formulated at 60 mg/kg in 0.5% w/v methyl cellulose. Both drugs were administered by oral gavage once a day, with 5 days on 2 days off regimen. In the combination group, ABT-263 was administered first and AZD-1775 90 minutes later. Treatments were administered for 4 weeks. Tumor volume and body weight were measured twice a week. Animals were sacrificed at 4 weeks, when tumors exceeded 1,000 mg or when animal health was compromised.

Results

Neuroendocrine carcinomas from different tissues of origin share gene expression programs

To identify potential therapeutic targets in SCNPC, we began by defining transcriptional programs associated with neuroendocrine carcinomas across tissue types. We evaluated whole-transcriptome RNA-sequencing (RNA-seq) data that included 25 prostate cancer metastases from a previously published study (17) and newly generated RNA-seq data from 91 castration-resistant prostate cancer (CRPC) metastases, eight MCC patient tumors, and 12 SCLC metastases or cell lines. We first subclassified the CRPC tumors using a published 10-gene signature of AR program activity and a 10-gene signature associated with neuroendocrine differentiation (ref. 13; **Fig. 1A**). We designated AR⁺/neuroendocrine⁻ tumors as AR program-active prostate cancers (ARPCs), and AR⁻/neuroendocrine⁺ tumors as SCNPCs. Merkel cell carcinoma and SCLC exhibited high expression of neuroendocrine signature genes in concordance with samples in the SCNPC category and did not express AR signature genes (**Fig. 1A**). On a global level, multidimensional scaling analysis demonstrated that MCC and SCLC cluster with SCNPC samples and away from ARPCs (**Fig. 1B**). We next sought to identify additional genes that distinguish SCNPC from ARPCs and found 817 genes with high consistent differential expression ($|\log_{2}FC| \geq 4$ and $FDR < 0.05$) that also displayed similar expression across MCC and SCLC (**Fig. 1C**). Disregarding the level of differential expression, 4,988 out of 20,413 genes measured by RNA-seq distinguished ARPC from neuroendocrine-associated cancers ($FDR < 0.05$). The genes commonly upregulated in the neuroendocrine-associated cancers are enriched for KEGG pathways associated with neuronal and cell-cycle-related pathways (Supplementary Fig. S1A).

The gene expression programs associated with neuroendocrine differentiation comprise both unique effector proteins that carry out specialized cell functions and transcription factors (TFs) that regulate the activity of downstream networks dictating cell identity. We next sought to determine which shared TFs across neuroendocrine tumors may drive a common cell fate and potentially regulate shared therapeutic targets. To meet the criteria of a TF, we combined human TFs from the Functional Annotation of the Mammalian Genome (FANTOM5) database with all proteins that were associated with "TF activity" in the AmiGO database. Of the 4,988 conserved

**Figure 1.**

Neuroendocrine carcinomas originating in different organ sites share gene expression programs. **A**, Heatmap showing relative transcript levels measured by RNA sequencing of AR-signature genes and neuroendocrine signature genes across patient metastases and cancer cell lines. **B**, Multidimensional scaling analysis of samples across all genes measured by RNA-sequencing. **C**, Heatmap of top 817 differentially expressed genes (|log₂ fold change| >4, FDR 0.05) in SCNPC versus ARPC tumors with expression shown across all samples. **D**, Heatmap of the expression 444 transcription factors differentially expressed between ARPCs and neuroendocrine cancers. **E–G**, Expression levels of MYC family members across tumor subtypes determined by RNA-seq (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$ by Student *t* test).

neuroendocrine-associated genes, 444 met the TF criteria. Notably, the most highly expressed TFs include several known to regulate neuronal or neuroendocrine differentiation programs, including SOX2, NEUROD1, and HES6 (Fig. 1D). The most differentially upregulated TF between ARPCs and neuroendocrine tumors, ST18, is associated with neuronal differentiation and is highly expressed in endocrine cells in the pancreas. Another highly upregulated TF, MYT1L, has been implicated in the maintenance of neuroendocrine differentiation (18) and can reprogram fibroblasts into neurons when coexpressed with BRN2 and ASCL1 (19). Elevated MYT1L is correlated with poor prognosis in patients with medulloblastoma (20), another small-cell tumor type.

Members of the MYC family of TFs are commonly altered in prostate cancer. Consistent with previous findings, MYC is expressed highly in ARPCs (Fig. 1E) whereas MYCN is elevated in all three neuroendocrine tumor types profiled relative to ARPC (Fig. 1F). We found that MYCL is also highly expressed across the three neuroendocrine tumor types relative to ARPC (Fig. 1G) and has recently been implicated in the initiation of SCLC (21).

Long noncoding RNAs (lncRNAs) are typically lowly expressed but are highly tissue specific. The expression of lncRNAs has been associated with prognosis in some cancers, including small-cell neuroendocrine cancers. Several lncRNAs, such as HOTAIR and Schlap-1, are associated with prostate cancer proliferation and invasion (22, 23). We derived a list of lncRNAs and pseudogenes from ENSEMBL and NCBI GRCh38 annotations and determined that 447 out of 2,283 lncRNAs and pseudogenes quantitated by RNA-seq are upregulated across SCNPC, SCLC, and MCC relative to ARPCs (Supplementary Fig. S1B). As the majority of lncRNAs have unknown functions, we conducted a guilt-by-association analysis for the top 50 most highly expressed neuroendocrine-associated lncRNAs (24). These 50 lncRNAs have high correlation to protein-coding genes associated with KEGG pathways related to neuronal processes and prostate cancer (Supplementary Fig. S1C).

Recent studies profiling the temporal alterations in gene expression during transitions from ARPC to SCNPC identified several hundred lncRNAs associated with the acquisition of the SCNPC phenotype (25). Notably, the expression of several lncRNAs is associated with the rapid development of metastasis following androgen deprivation therapies (ADT). We found a high degree of overlap between the lncRNAs upregulated across the neuroendocrine cancers we evaluated and those explicitly associated with prostate cancer transdifferentiation: of 122 transcripts comprising a lncRNA SCNPC signature, 100 (80%; $P < 0.0001$) were differentially regulated across SCLC and MCC. Although lncRNAs may serve as markers of neuroendocrine differentiation and have utility in predicting treatment outcomes, they are currently challenging to exploit as therapeutic targets.

Profiling the druggable genome across neuroendocrine tumors identifies therapeutic targets in SCNPC

To identify potential conserved dependencies on druggable targets among neuroendocrine tumors, we profiled the expression of genes comprising the druggable genome (26). Of 4,026 druggable targets measured, 875 had similar expression across neuroendocrine tumors relative to ARPCs (Fig. 2A). A subset of druggable targets is represented by cell surface proteins expressed uniquely on subtypes of neoplastic cells that can be exploited for immune-based therapy and for antibody or small-molecule delivery of targeted therapeutics. Notably, the Notch ligand receptor DLL3 was among the top differentially upregulated cell surface proteins between ARPCs and the neuroendocrine tumors (Fig. 2A and B). DLL3 has previously been

shown to be upregulated in SCLC. An antibody–drug conjugate (ADC), Rovalpituzumab tesirine, has demonstrated clinical efficacy toward SCLCs and is currently being investigated in clinical trials (27). As with the human tumors, we found that DLL3 expression was upregulated in SCNPC PDX models compared with ARPC PDX models (Fig. 2C). These data suggest that therapeutics targeting DLL3 may be broadly effective across neuroendocrine tumors including SCLC, SCNPC, and MCC.

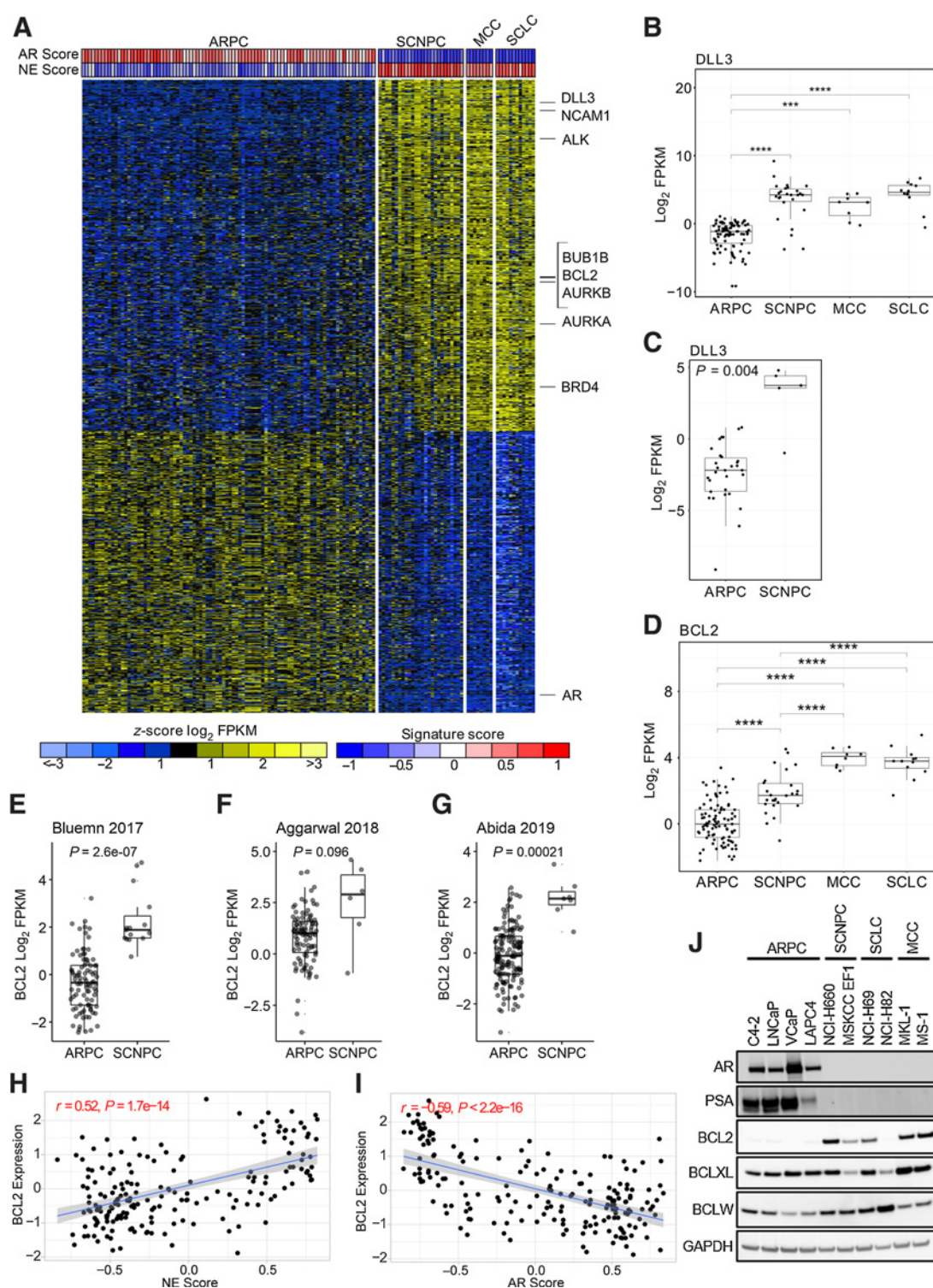
Additional targets that exhibited differential upregulation in neuroendocrine tumors relative to ARPCs include aurora kinase A and B (AURKA, AURKB), the anaplastic lymphoma kinase A (ALK), and the bromodomain and extraterminal domain family member BRD4 (Fig. 2A). Recent studies have demonstrated the efficacy of BET inhibitor therapy in preclinical models of SCLC with downregulation of the neuronal lineage TF ASCL1 and the induction of apoptosis (28). Furthermore, BET inhibitors have been shown in preclinical studies to be particularly effective in MYC-driven cancers such as neuroblastoma (29), which is also a feature of SCNPCs where MYCN and MYCL are highly expressed.

BCL2 is a therapeutic target in SCNPC

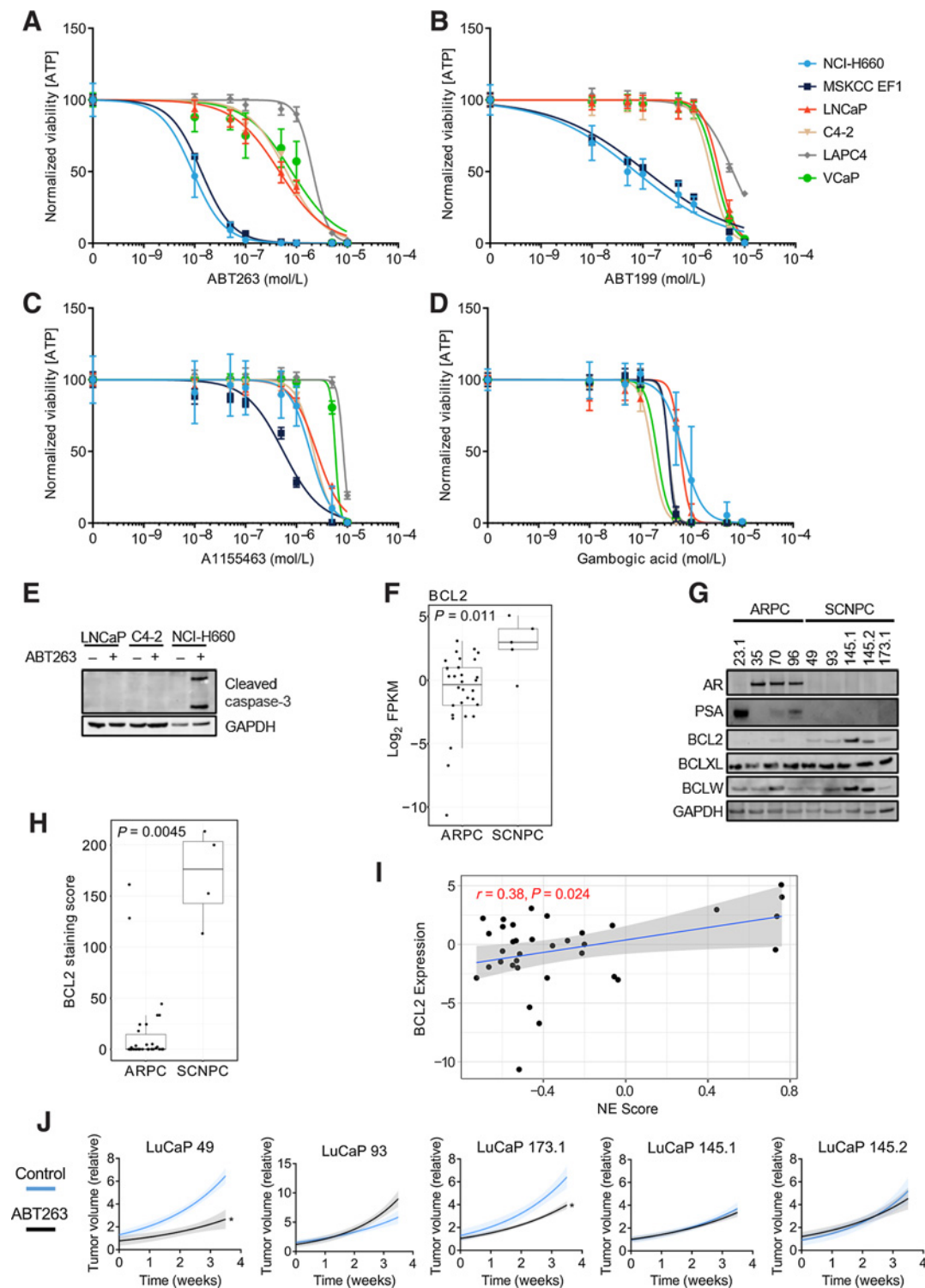
Transcripts encoding the antiapoptotic protein BCL2 were among the most highly expressed druggable targets across neuroendocrine carcinomas (Fig. 2A and D). We chose to focus on BCL2 as both MCC and subsets of SCLC have previously been shown to be sensitive to BCL2 family inhibitors but their effects have not been described in SCNPC (30, 31). We determined that other frequently cotargeted members of the BCL2 family, BCLXL and BCLW, were not differentially upregulated in SCNPC (Supplementary Fig. S2A and S2B). BCL2 expression was significantly higher in SCNPCs versus ARPCs in three independent studies of metastatic CRPC (Fig. 2E–G; refs. 13, 32, 33). BCL2 expression is also significantly positively correlated with the 10-gene neuroendocrine signature score and significantly negatively correlated with AR activity (Fig. 2H and I). BCLXL expression has no significant correlation with either signature score and BCLW expression is positively correlated with AR activity and negatively correlated with the neuroendocrine signature (Supplementary Fig. S2C–S2F).

We next assayed BCL2 family protein levels across a panel of cell lines that include ARPC, represented by LNCaP, C4-2, LAPC4 and VCaP, and SCNPC, represented by the NCI-H660 and MSKCC EF1 lines. Merkel cell carcinoma lines are designated MKL-1 and MS-1. SCLC lines are NCI-H69, NCI-H82. In agreement with the tumor-derived transcript data, BCL2 protein is highly expressed across the majority of the neuroendocrine cell lines, including NCI-H660, and was not detectable in the ARPC lines (Fig. 2J).

We next assessed the responses to pharmacologic inhibitors of BCL2 family proteins across ARPC and SCNPC cells. We determined that SCNPC NCI-H660 and MSKCC EF1 cells are substantially more sensitive to the pan BCL-XL, BCL-W, and BCL2 inhibitor ABT-263, and the more selective BCL2 inhibitor, ABT-199, than other prostate lines profiled (Fig. 3A and B). For example, the approximate IC_{50} concentrations of ABT-199/venetoclax in NCI-H660 versus the ARPC LNCaP are 50 nmol/L versus 500 nmol/L, respectively. In addition, NCI-H660 and MSKCC EF1 have comparable sensitivity to other prostate lines when treated with the more selective BCL-XL inhibitor A115463 (Fig. 3C) or the more selective BCL-W inhibitor, gambogic acid (Fig. 3D). A 24-hour treatment with 100 nmol/L ABT-263 is sufficient to induce cell death in NCI-H660 SCNPC cells, as evidenced by cleaved caspase-3 whereas ARPCs showed no evidence of an apoptotic response (Fig. 3E). We also profiled the response of this

**Figure 2.**

Analyses of the druggable genome identify BCL2 as a candidate therapeutic target in SCNPC. **A**, Heatmap displaying genes in the druggable genome with differential expression between ARPCs and neuroendocrine tumors. **B**, Expression of the transcript encoding the cell surface notch pathway gene DLL3 in human tumors and (C) patient-derived xenografts (PDX). **D**, Expression of transcripts encoding BCL2 across tumor subtypes. **E–G**, Boxplots depicting BCL2 transcript levels measured by RNAseq in metastatic tumors from patients with CRPC. **H** and **I**, Pearson correlations between the neuroendocrine (NE) or androgen receptor (AR) signature score and gene expression. **J**, Western blot analysis of selected BCL2 family members across a panel of cell lines. (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$ by Student *t* test).

**Figure 3.**

BCL2 antagonists inhibit the growth of SCNPC. **A–D**, Dose response curves of BCL2-family inhibitors across a panel of prostate cancer cell lines. NCI-H660 and MSKCC EF1 are SCNPC, and LNCaP, C4-2, LAPC4, and VCaP are ARPC. **E**, Western blot for cleaved caspase-3 following 24-hour treatment with ABT-263 across the indicated cell lines. **F**, BCL2 expression in ARPC versus SCNPC PDX models determined by RNAseq. $P = 0.011$ by Student t test. **G**, Western blot of selected BCL2 family members across a panel of prostate cancer PDX models. **H**, Quantification of BCL2 staining of LuCaP TMA. **I**, Scatterplot depicting Pearson correlation between the neuroendocrine (NE) signature score and *BCL2* gene expression in PDX lines. **J**, Normalized tumor volumes of PDX models treated with vehicle control or ABT-263. *, $P < 0.05$.

prostate cancer cell line panel to older generation BCL2 inhibitors sabutoclax, obatoclax, and a selective MCL1 inhibitor, A1210477. However, NCI-H660 was equally or less sensitive to these compounds than other prostate cancer cell lines (Supplementary Fig. S3A–S3D). In agreement with the pharmacologic studies, siRNA knockdown of BCL2 decreased viability of NCI-H660 but not LNCaP cells (Supplementary Fig. S3E–S3H).

To further assess the efficacy and specificity of targeting BCL2 in SCNPC, we evaluated the effects of pharmacologic BCL2 inhibition on PDX models of SCNPC. RNA-seq confirmed that SCNPC PDX tumors expressed significantly higher levels of BCL2 transcripts compared with ARPC PDX tumors (Fig. 3F). These findings were confirmed by Western blot analysis of BCL2 protein in a panel of prostate cancer PDX tumors: BCL2 protein was highly expressed in five of five SCNPC PDX lines, whereas three of four ARPC PDX lines had no detectable BCL2 and one expressed low levels (Fig. 3G). IHC analysis confirmed high BCL2 expression in SCNPC relative to ARPC tumors with the majority of ARPCs having no detectable staining (Fig. 3H). Mirroring the patient tumors, BCL2 expression levels in PDX models are positively correlated with the neuroendocrine signature score (Fig. 3I).

We treated five castration-resistant SCNPC PDX models, LuCaP49, LuCaP93, LuCaP173.1, LuCaP145.1, and LuCaP145.2, with the BCL2 antagonist ABT-263/Navitoclax. Compared with vehicle control, ABT-263 significantly reduced the growth of two lines, LuCaP49 and LuCaP173.1, for an overall response rate of 40% (Fig. 3J). Notably, LuCaP49 PDX tumor volumes at the 4-week end of study evaluation were more than four times smaller for ABT-263 treatment than vehicle control ($P = 0.05$ by Student t test).

Targeting the Wee1 kinase with BCL2 inhibition represses SCNPC growth

Although the *in vitro* studies indicated that neuroendocrine carcinomas cells are sensitive to BCL2 antagonism and two *in vivo* PDX models of SCNPC confirmed the antitumor activity of BCL2 inhibition, we determined that three SCNPC PDX lines were resistant to ABT-263 (Fig. 3J). BCL2 levels in the resistant PDX tumors approximated the levels of BCL2 in the responsive tumors (Fig. 3G). We evaluated protein levels of other BCL2 family members that may contribute to resistance to ABT-263 in the PDX tumors; however, no single protein was able to distinguish responding versus nonresponding models (Supplementary Fig. S3I). We next evaluated the druggable genome data for additional differentially expressed targets with available pharmacologic inhibitors. These analyses determined that the mitotic checkpoint kinase Wee1 is differentially upregulated in neuroendocrine carcinomas versus ARPCs (Fig. 4A–D). Wee1 expression was negatively associated with AR activity in prostate cancer metastases and PDX lines and positively associated with neuroendocrine activity in prostate cancer PDX models (Fig. 4E–I). We confirmed that Wee1 protein is highly expressed in SCNPC cell lines and PDX models compared with ARPC models, as assessed by Western blot and IHC (Fig. 4J and K; Supplementary Fig. S4A and S4B). Notably, Wee1 inhibition has demonstrated efficacy in neuroblastoma, SCLC, medulloblastoma, and other neuroendocrine-associated malignancies (34–38).

We evaluated the effects of the Wee1 antagonist AZD-1775 across the SCNPC PDX models and SCNPC cell lines. As a single agent, AZD-1775 has minimal activity toward SCNPC cell lines *in vitro* though more substantial effects toward ARPC lines (Supplementary Fig. S5A–S5D). We next combined AZD-1775 with ABT-263. These drugs exhibited synergy in the SCNPC lines with a

combination index score of 0.556 in NCI-H660 (Supplementary Fig. S5E and S5F) while demonstrating at best additive effects in the ARPC lines tested (Supplementary Fig. S5G and S5H). To assess on-target Wee1 inhibition, we measured the phosphorylation of downstream kinase CDK1 and confirmed that exposure to AZD-1775 reduced the inhibitory phosphorylation of this key regulator of mitosis (Supplementary Fig. S4C–S4E). Consistent with the *in vitro* results, only very modest single-agent AZD-1775 activity was observed in two SCNPC PDX models with three showing no responses (Fig. 4N). The combination of ABT-263 and AZD-1775 significantly inhibited the growth of four of five SCNPC PDX lines (80% response rate). Notably, the ABT-263-resistant LuCaP93 SCNPC line demonstrated marked sensitivity to the drug combination with average end of study tumor volumes four times larger in control or AZD-1775 treatment arms versus AZD-1775 plus ABT-263 (Fig. 4N; $P = 0.017$, Student t test).

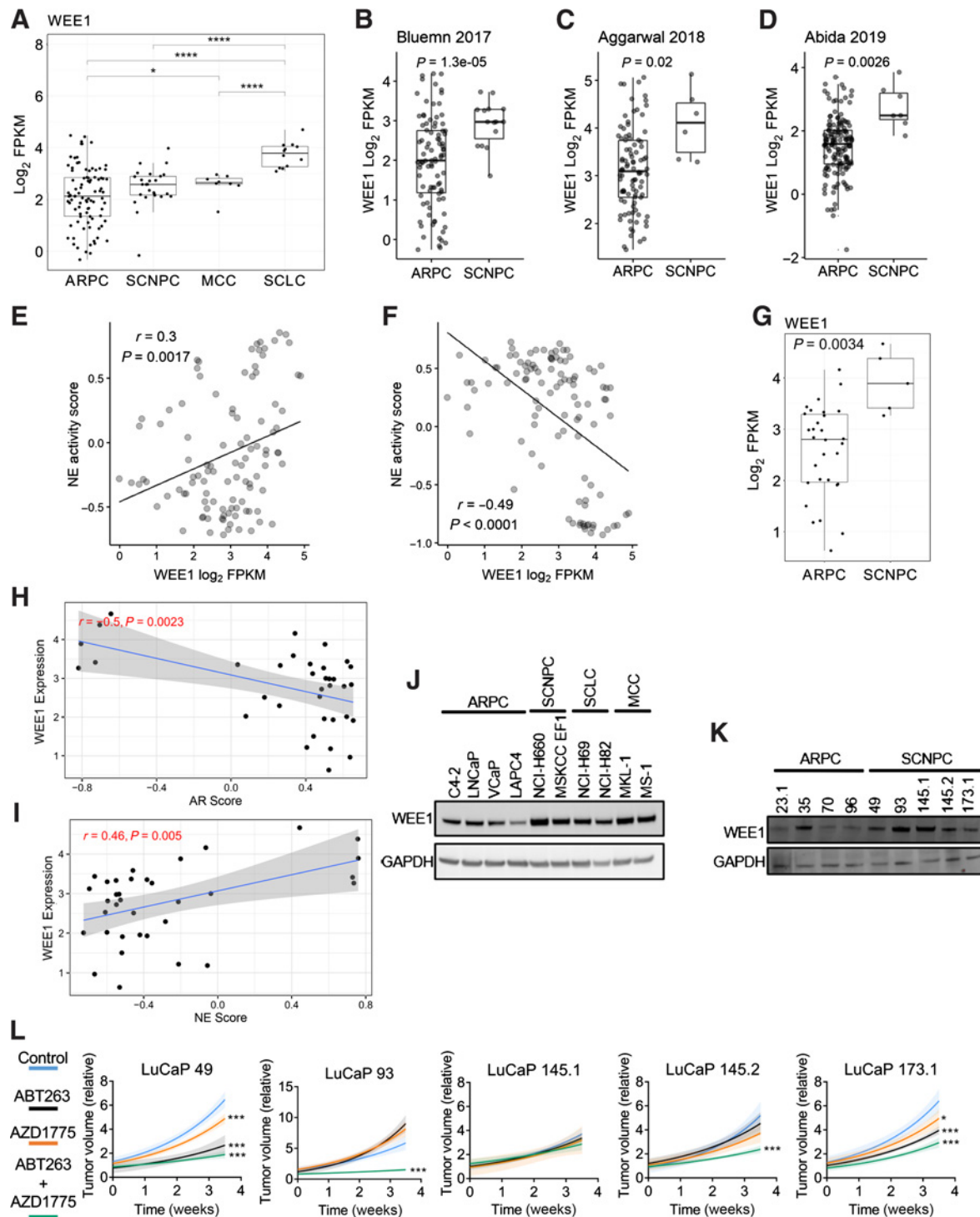
Discussion

The emergence of therapy-associated neuroendocrine differentiation in patients with metastatic prostate cancer portends a short survival with very limited benefit from current therapeutics. These poor outcomes are mirrored by low survival rates in other neuroendocrine tumors such as SCLC and metastatic MCC. Notably, these malignancies share a number of oncogenic features that include loss of TP53 and RB1 tumor suppressor function and gain of MYC activity. In this study, we identified additional phenotypic similarities that include a large number of coexpressed lncRNAs, TFs that influence neuronal and neuroendocrine activity, and cell surface proteins that represent viable therapeutic targets. As the majority of SCNPCs included in this study were associated with treatment, future studies are required to confirm concordance with *de novo* SCPC.

MYCN is perhaps the most notable TF proposed to drive neuroendocrine cancers arising in diverse organs including neuroblastoma, SCLC, and SCNPC where it may function to promote lineage switching (39, 40). We found that MYCL, another MYC family member recently implicated in the initiation of SCLC (21), is also highly expressed in MCC and SCNPC. In addition, we identified 373 TFs with expression patterns conserved across MCC, SCLC, and SCNPC. Whether these factors are driving the neuroendocrine phenotype or are merely a consequence of it, remains to be explored.

lncRNAs represent a class of transcripts attracting attention for their roles in promoting the malignant features of some tumors. We identified 285 lncRNAs with conserved expression patterns across MCC, SCLC, and SCNPC. Of the most highly expressed pan-neuroendocrine-associated lncRNAs we identified, MIAT and LINC01346 have high correlation with the expression of genes involved in the KEGG prostate cancer pathway. MIAT has previously been identified as highly expressed in SCNPC; however, LINC01346 has no previously described connection to prostate cancer. The elevated expression of MIAT and LINC01346 and their pan-neuroendocrine correlation suggest that it may also play a role in supporting the growth or maintenance of these tumors.

The identification of cancer-specific cell surface molecules is increasingly relevant with the advent of successful chimeric antigen receptor T-cell therapies and ADCs. The ADCs directed toward the cell surface protein DLL3 have produced responses in patients with SCLC and SCNPC (27, 41). CEACAM5 was recently identified as a cell surface antigen selectively enriched in SCNPC (42). Although

**Figure 4.**

WEE1 inhibition can enhance the effects of BCL2 antagonism in SCNPC. **A**, Boxplot of WEE1 expression across patient metastases and cell lines. *, $P < 0.05$; ****, $P < 0.0001$ by Student t test. **B–D**, Boxplots showing WEE1 transcript expression measured by RNA-seq in metastatic CRPCs in three independent studies. **E** and **F**, Scatterplots depicting Pearson correlation between WEE1 transcript levels and neuroendocrine signature score, or AR signature score. **G**, Boxplot of WEE1 expression in ARPC and SCNPC PDX models of prostate cancer (P value from Student t test). **H** and **I**, Scatterplots depicting Pearson correlation between WEE1 transcript levels and neuroendocrine signature score, or AR-signature score. **J** and **K**, Western blots showing WEE1 protein levels across cell lines and LuCaP PDX models, respectively. **L**, Normalized tumor volumes of PDX models treated with vehicle control, ABT-263, AZD-1775, or the combination of ABT-263 and AZD-1775 or vehicle (bottom; *, $P < 0.05$; ***, $P < 0.001$).

CEACAM5 is highly expressed in SCNPC and SCLC, we found it to be lowly expressed in MCC and therefore did not fall into our list of pan-neuroendocrine-associated genes.

We identified several potential druggable targets spanning SCNPC, SCLC, and MCC that included the antiapoptotic protein BCL2. Elevated BCL2 protein expression has been identified in multiple neuroendocrine tumor types, although not specifically SCNPC, and BCL2 inhibition has recently been shown to induce cell death in MCC and SCLC cell lines with high levels of BCL2 protein (30, 31). A generalized model to explain high levels of antiapoptotic BCL2 family member expression in many cancers has been proposed in which a cell undergoing oncogenic transformation will be subjected to higher apoptotic stress and upregulation of proapoptotic BCL2 family members; thus, the cell needs to acquire compensatory alterations to overcome apoptotic cues. In lymphomas, MYC amplifications are often seen in conjunction with BCL2 amplification and in neuroblastoma, MYCN amplification is correlated with higher BCL2 levels (43). In prostate cancer, the AR has been shown to directly repress BCL2 transcription and consequently AR antagonist therapy results in elevated BCL2 (44, 45).

Although BCL2 has long been viewed as an attractive therapeutic target, only recently have inhibitors been developed with potent anti-BCL2 activity and tolerable side effect profiles. The BCL-2 antagonist venetoclax was recently approved as a treatment for chronic lymphocytic leukemia and acute myelogenous leukemia, and clinical trials in other tumor types are underway. Recent studies demonstrated effects of BCL2 inhibition in preclinical models of AR-low prostate cancers and in combination with AR blockade in AR-active preclinical models (45). Although the combination of BCL2 and Wee1 inhibition is novel, it has previously been proposed that BCL2 family proteins can regulate sensitivity to antimetabolic agents (46, 47). CDK1, negatively regulated by Wee1, phosphorylates and inactivates antiapoptotic BCL2 family members, including BCL2, during prolonged mitosis leading to cell death (48, 49). It is our hypothesis that further inhibition of BCL2 results in the synergistic effects of AZD-1775 and ABT-263. Our studies support testing BCL2 inhibitors in patients with AR-null SCNPC and evaluating additional therapeutics such as Wee1 antagonists that could synergize with BCL2-directed therapeutics in SCNPCs that resist single-agent treatment.

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E. Corey reports receiving commercial research grants from Abbvie and AstraZeneca. P.S. Nelson is an employee/paid consultant for Astellas and Janssen. No potential conflicts of interest were disclosed by the other authors.

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Regulation of CEACAM5 and Therapeutic Efficacy of an Anti-CEACAM5-SN38 Antibody-drug Conjugate in Neuroendocrine Prostate Cancer

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ABSTRACT

Purpose: Neuroendocrine prostate cancer (NEPC) is an aggressive form of castration-resistant prostate cancer (CRPC) for which effective therapies are lacking. We previously identified carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5) as a promising NEPC cell surface antigen. Here we investigated the scope of CEACAM5 expression in end-stage prostate cancer, the basis for CEACAM5 enrichment in NEPC, and the therapeutic potential of the CEACAM5 antibody-drug conjugate labetuzumab govitecan in prostate cancer.

Experimental Design: The expression of CEACAM5 and other clinically relevant antigens was characterized by multiplex immunofluorescence of a tissue microarray comprising metastatic tumors from 34 lethal metastatic CRPC (mCRPC) cases. A genetically defined neuroendocrine transdifferentiation assay of prostate cancer was developed to evaluate mechanisms of CEACAM5 regulation in NEPC. The specificity and efficacy of labetuzumab govitecan was

determined in CEACAM5⁺ prostate cancer cell lines and patient-derived xenografts models.

Results: CEACAM5 expression was enriched in NEPC compared with other mCRPC subtypes and minimally overlapped with prostate-specific membrane antigen, prostate stem cell antigen, and trophoblast cell surface antigen 2 expression. We focused on a correlation between the expression of the pioneer transcription factor *ASCL1* and *CEACAM5* to determine that *ASCL1* can drive neuroendocrine reprogramming of prostate cancer which is associated with increased chromatin accessibility of the *CEACAM5* core promoter and CEACAM5 expression. Labetuzumab govitecan induced DNA damage in CEACAM5⁺ prostate cancer cell lines and marked antitumor responses in CEACAM5⁺ CRPC xenograft models including chemotherapy-resistant NEPC.

Conclusions: Our findings provide insights into the scope and regulation of CEACAM5 expression in prostate cancer and strong support for clinical studies of labetuzumab govitecan for NEPC.

Introduction

While androgen deprivation therapy (ADT) is initially effective for the treatment of hormone-sensitive prostate adenocarcinoma, resistance is inevitable and leads to a state known as castration-resistant prostate cancer (CRPC). CRPC is heterogeneous and comprises multiple molecular phenotypes that diverge from conventional prostate adenocarcinoma and include neuroendocrine prostate cancer (NEPC) which is a high-grade, poorly differentiated, and lethal neuroendocrine carcinoma with no effective treatments. NEPC accounts for up to 20% of lethal metastatic CRPC (mCRPC) and exhibits rapid metastatic dissemination, loss of androgen receptor

(AR) signaling, and expression of neuroendocrine differentiation markers. NEPC rarely arises *de novo* and primarily emerges from prostate adenocarcinoma through a process of neuroendocrine transdifferentiation as an adaptive response to the selective pressure of ADT (1).

While an understanding of the determinants of neuroendocrine transdifferentiation of prostate cancer remains incomplete, several genetic alterations have been associated with progression to NEPC. These include loss of the tumor suppressor genes *RB1* and *TP53*, amplification or overexpression of *MYCN* and *AURKA*, and activation of the PI3K/AKT pathway (2, 3). These genetic derangements are also common to poorly differentiated neuroendocrine cancers arising from other epithelial tissues including the lung. In genetically engineered mouse models, combined loss of *Rb1*, *Trp53*, and *Pten* in the prostate promotes the development of tumors displaying castration resistance, lineage plasticity, and a neuroendocrine cancer phenotype (4, 5). Human prostate epithelial transformation models have also underscored the importance of these genetic perturbations in the initiation of NEPC (6, 7). Yet neuroendocrine transdifferentiation does not appear to be an obligate outcome of these genetic events in human prostate cancer (8), indicating that other factors may be involved.

In general, NEPC represents an epigenetic cancer state distinct from prostate adenocarcinoma with unique patterns of DNA methylation, chromatin accessibility, and epigenetic regulator expression (6, 9, 10). However, NEPC can vary in histologic appearance and neuroendocrine marker expression, likely due to molecular heterogeneity. Small-cell lung cancer (SCLC) shares many phenotypic characteristics with NEPC. Recently, four molecular subtypes of SCLC have been

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Translational Relevance

Neuroendocrine prostate cancer (NEPC) is an aggressive subtype of castration-resistant prostate cancer without effective treatments. Here we examined the expression of carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5) compared with other relevant prostate cancer antigens in a series of lethal, metastatic prostate cancers. CEACAM5 is preferentially expressed in NEPC and tumor expression appears to correlate with serum CEA levels in NEPC cases. Through functional genomics studies, we illustrate the potential role of the pioneer transcription factor achaete-scute homolog 1 (ASCL1) in the epigenetic regulation of CEACAM5 expression and neuroendocrine transdifferentiation of prostate cancer. Finally, we redirect the anti-CEACAM5–SN38 antibody–drug conjugate, labetuzumab govitecan, for preclinical studies in prostate cancer and demonstrate tumor eradication in multiple xenograft models of CEACAM5⁺ prostate cancer including NEPC. Overall, we describe the scope of CEACAM5 expression in end-stage prostate cancer, report a mechanism of CEACAM5 gene regulation by ASCL1, and provide evidence to support imminent clinical investigation of labetuzumab govitecan in men with CEACAM5⁺ NEPC.

identified, of which two are marked by differential expression and activity of the pioneer neural basic helix-loop-helix transcription factors achaete-scute homolog 1 (ASCL1) and neurogenic differentiation factor 1 (NeuroD1; ref. 11). In a mouse model of SCLC driven by *Rb1* and *Trp53* loss, *Ascl1* but not *Neurod1* was required for the initiation of SCLC (12). NeuroD1^{high} SCLC appears to progress from an ASCL1^{high} SCLC state through a process mediated by enhanced MYC expression (13). Given the biological parallels between SCLC and NEPC, these lineage-defining transcription factors may also be operative in NEPC.

The expression of cell surface proteins reflects specific cellular lineage programs in normal development and in cancer. The development of targeted therapies directed against prostate cancer cell surface antigens is an active area of research that must account for the heterogeneity of CRPC phenotypes reflecting diverse cancer differentiation states. Using a systematic approach, we previously identified expression of the human carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5, also known as CEA) in a large subset of NEPC (14). CEACAM5 is a cell surface protein that is upregulated in a variety of other human epithelial malignancies including colorectal cancer (15) and has been functionally associated with tumor differentiation, invasion, and metastasis (16, 17). Multiple therapeutic approaches to target CEACAM5 in cancer are in development including vaccines, bispecific T-cell engagers, chimeric antigen receptor T-cell therapies, and antibody–drug conjugates (ADCs). Labetuzumab govitecan (IMMU-130) is a CEACAM5 ADC composed of a humanized CEACAM5 mAb named labetuzumab conjugated to the potent topoisomerase I inhibitor 7-ethyl-10-hydroxycamptothecin (SN-38) via a unique hydrolysable linker (CL2A; ref. 18). SN-38 is the active metabolite of irinotecan which is commonly used as chemotherapy for colorectal and pancreatic cancer (19). Labetuzumab govitecan has demonstrated activity in preclinical models of colorectal cancer (18, 20) as well as safety and potential efficacy in a phase I/II clinical trial in patients with treatment-refractory metastatic colorectal cancer (21). However, labetuzumab govitecan has yet to be evaluated in the treatment of prostate cancer.

Here we characterize CEACAM5 expression in end-stage mCRPC relative to other cell surface antigens that are the active clinical focus of diagnostic and therapeutic development. We investigate the molecular basis for CEACAM5 expression in NEPC and uncover insights into the cancer differentiation-specific regulation of CEACAM5. Finally, we evaluate the antitumor activity of labetuzumab govitecan in preclinical models of CEACAM5⁺ CRPC, including NEPC, to justify the clinical investigation of this therapeutic agent in prostate cancer.

Materials and Methods

Cell lines

DU145 (catalog No. DU-145, RRID:CVCL_0105), 22Rv1 (catalog No. CRL-2505, RRID:CVCL_1045), C4-2B (catalog No. CRL-3315, RRID:CVCL_4784), and NCI-H660 (catalog No. CRL-5813, RRID:CVCL_1576) cell lines were purchased from the ATCC and LNCaP95 were a gift from Stephen R. Plymate (University of Washington, Seattle, WA). All cell lines were validated by short tandem repeat analysis after receipt. DU145, 22Rv1, C4-2B, and MSKCC EF1 (derived from the organoid line MSKCC-CaP4) were maintained in RPMI medium supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, and 4 mmol/L GlutaMAX. NCI-H660 cells were maintained in Advanced DMEM/F12 medium supplemented with B27, 4 mmol/L GlutaMAX, and 10 ng/mL recombinant human basic FGF (bFGF) and EGF. Cell lines were cultured no more than 3 weeks after thawing prior to use in described experiments.

mIF of tissue microarrays

UW mCRPC TAN TMA (Prostate Cancer Biorepository Network) and FDA normal organ TMA (US Biomax Inc.) were used for mIF studies (Supplementary Tables S1–S3). Slides were stained on a Leica BOND Rx stainer (Leica) using Leica Bond reagents for antigen retrieval, antibody stripping (Epitope Retrieval Solution 2), and rinsing after each step (Bond Wash Solution). A high stringency wash was performed after the secondary and tertiary applications using high-salt TBST solution (0.05 mol/L Tris, 0.3 mol/L NaCl, and 0.1% Tween-20, pH 7.2–7.6). Opal Polymer HRP Mouse plus Rabbit (PerkinElmer) was used for all secondary applications.

H-scoring of CEACAM5 expression

H-scores were generated from the CEACAM5 mIF data using the CytoNuclear LC v2.0.6 module and HALO software. Briefly, individual cells were classified as having negative, weak, moderate, or strong CEACAM5 staining and assigned intensity scores of 0, 1, 2, and 3, respectively. The intensity score ranges were defined on the basis of CEACAM5 fluorescent intensity values as follows: 0 = 0 – positive CEACAM5 threshold value, 1 = threshold value – 25th quartile median, 2 = 25th quartile median – 75th quartile median, and 3 = 75th quartile median – maximum value reported. Intensity scores were then multiplied by the percentage of stained cells for a range of 0–300.

Serum CEA quantification

Cryopreserved serum samples obtained at rapid autopsy or a patient visit prior to rapid autopsy were obtained from the University of Washington Tissue Acquisition Necropsy (UW TAN) repository. CEA quantification was performed using a Clinical Laboratory Improvement Amendments–licensed CEA ELISA test (University of Washington Research Testing Services).

Exome sequencing analysis

Paired-end exome sequencing [next-generation sequencing (NGS)] was performed using Illumina HiSeq or Illumina NovaSeq on genomic DNA isolated from rapid autopsy tissue samples. Sequence reads were aligned to the human reference genome hg19 using the Burrows-Wheeler Aligner (RRID:SCR_010910). GATK (RRID:SCR_001876) best practice was adopted to process all aligned BAM files. Germline and somatic mutation analyses were performed using HaplotypeCaller and Mutect2. All detected mutations were annotated using ANNOVAR hg19 (RRID:SCR_012821) and manual curation was performed before determination of pathogenicity. Copy number was derived following the standardized Sequenza pipeline (RRID:SCR_016662). All copy-number calls were manually curated for potentially missed mid-sized structural aberrations (15–50 nt indels).

C4-2B neuroendocrine transdifferentiation assay

C4-2B cells were seeded in 6-well tissue culture plates at a density of 10^5 cells per mL in 3 mL of RPMI medium supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, and 4 mmol/L GlutaMAX. Cells were transduced approximately 4–6 hours after seeding at a defined multiplicity of infection of 4 for each lentivirus. Seventy-two hours after transduction, cells were trypsinized, washed, and transferred to 100-mm tissue culture plates in 15 mL of Advanced DMEM/F12 medium supplemented with B27, 4 mmol/L GlutaMAX, and 10 ng/mL recombinant human bFGF and EGF. Media were replenished every 3–4 days. Cells were collected 11 days posttransduction for analysis.

Assay for transposase-accessible chromatin sequencing

Briefly, 50,000 cells were lysed in buffer containing NP-40, Tween-20, and digitonin. Nuclei were collected after centrifugation and transposed with Tn5 transposase for 30 minutes at 37°C. DNA was purified by MinElute Reaction Cleanup Kit (Qiagen) followed by PCR amplification to append indices/adapters, library purification, and quality control by Agilent TapeStation and library quantitation by qPCR. Assay for transposase-accessible chromatin sequencing (ATAC-seq) libraries underwent paired-end 50 bp sequencing on an Illumina NovaSeq 6000. Raw reads were processed with the ENCODE ATAC-seq pipeline (22) for quality control, alignment by Bowtie 2 (RRID:SCR_005476), and peak calling by MACS2 (RRID:SCR_013291). Inferred transcription factor activity was determined by HINT-ATAC (23) using HOCOMOCO (RRID:SCR_005409) and JASPAR (RRID:SCR_003030) binding motifs.

ATAC qPCR

ATAC-qPCR targeting the *CEACAM5* core promoter peak was performed using ATAC libraries on the QuantStudio5 System (Thermo Fisher Scientific) with Applied Biosystems PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). The mean cycle threshold (C_t) obtained for each promoter region was normalized to the AK5 control primers (24).

Immunoblots

Whole cell extracts were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane using a transfer apparatus according to the manufacturer's instructions (Invitrogen). Membranes were blocked with 5% nonfat milk in PBST (DPBS + 0.5% Tween 20) for 30 minutes while shaking, then incubated with primary antibodies at 4°C for 16 hours. Membranes were washed three times for 5 minutes with PBST and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibody for 1 hour at room

temperature. Blots were washed three times for 5 minutes each with PBST and developed with Immobilon Western Chemiluminescent HRP Substrate (MilliporeSigma) for 3 minutes at room temperature. Blot images were acquired with a ChemiDoc Imaging System (Bio-Rad) or autoradiography film.

CEACAM5 surface protein detection by flow cytometry

DU145, 22Rv1, and MSKCC EF1 cells were dissociated with Versene-EDTA (Thermo Fisher Scientific) into single-cell suspensions. Cells were washed once with mAb wash buffer (MW; PBS + 0.1% FBS + 0.1% sodium azide) then resuspended in 100 µL MW and 5 µL of anti-CEACAM5-APC or IgG isotype-APC per 10^6 cells and incubated at room temperature in the dark for 30 minutes. Cells were washed once with MW, resuspended in MW, acquired on a BD FACSCanto II (BD Biosciences), and analyzed with FlowJo (v10; RRID:SCR_008520).

Labetuzumab cell surface binding

DU145, 22Rv1, and MSKCC EF1 cell lines expressing empty vector or CEACAM5 vector were dissociated nonenzymatically with Versene-EDTA into single-cell suspensions. Cells were washed once with PBS and resuspended in 100 µL of 1 µg/mL of h679 or labetuzumab (Immunomedics, Inc.) and incubated at 4°C on ice for 1 hour. Cells were then washed twice with PBS, incubated with an anti-human IgG-PE-Cy5 secondary antibody (Thermo Fisher Scientific) at 4°C on ice for 30 minutes, washed with PBS, acquired on a SH800 (Sony), and analyzed with FlowJo (v10).

γH2AX detection of dsDNA breaks

DU145, 22Rv1, and MSKCC EF1 cells were dissociated nonenzymatically with Versene-EDTA (Thermo Fisher Scientific), washed with PBS, resuspended in PBS and prechilled on ice at 4°C for 20 minutes, followed by incubation with labetuzumab, govitecan or h679-SN-38 (Immunomedics, Inc.), or SN-38 (Sigma) for 30 minutes on ice at 4°C. Cells were then washed six times with cold PBS, and cultured for 16 hours in culture media at 37°C. For extended SN-38-treated conditions, cells were cultured at 37°C in media containing SN-38 for 16 hours. Cells were then dissociated with trypsin 0.25%, washed with MW, fixed with BD Cytotoxicity Buffer (BD Biosciences), permeabilized with BD Phosflow Perm Buffer II (BD Biosciences), and stained with anti-γH2AX-BV421 or IgG isotype control, as per manufacturer's instructions. Cells were washed twice with MW, resuspended in MW, acquired on a BD FACSCanto II (BD Biosciences), and analyzed with FlowJo (v10).

SN-38 dose responses in prostate cancer cell lines

DU145, 22Rv1, MSKCC EF1, and NCI-H660 cells were seeded at 5×10^3 cells (50 µL) per well in 96-well flat bottom, tissue culture-treated, white plates (Corning). Cells were treated with serial dilutions of SN-38 (50 µL) in replicates of 8, diluted in appropriate culture media, at 37°C for 96 hours. Cell viability was determined using the CellTiter-Glo 2.0 Assay (Promega).

IHC of LuCaP patient-derived xenograft tumors

Formalin-fixed, paraffin-embedded tissue sections were baked at 65°C for 1–2 hours, deparaffinized in xylene, and rehydrated in 100%, 95%, and 70% ethanol. Tissue sections were heated in antigen retrieval buffer (0.2 mol/L citric acid and 0.2 mol/L sodium citrate) within a pressure cooker followed by PBS wash. Tissue slices were blocked with 2.5% horse serum for 30 minutes and then incubated with primary antibody diluted in 2.5% horse serum overnight at 4°C. HRP was

detected with ImmPRESS-HRP anti-mouse or anti-rabbit IgG peroxidase detection kits (Vector Laboratories) and staining was visualized with DAB peroxidase substrate (Dako). Tissue sections were counterstained with hematoxylin and dehydrated for mounting.

Mouse xenograft studies

All animal care and studies were performed in accordance with an approved Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee protocol and Comparative Medicine regulations. Six-week-old, male NSG (NOD-SCID-IL2R γ -null, RRID: BCBC_4142) mice were obtained from the Jackson Laboratory. A total of 5×10^6 cells from each prostate cancer cell line were suspended in 100 μ L of cold Matrigel (Corning) and implanted by injection subcutaneously into NSG mice. For LuCaP patient-derived xenografts (PDX), a 1-mm³ piece of prostate tumor tissue was surgically implanted subcutaneously into NSG mice. Mice were enrolled into a treatment arm when tumors reached 150 mm³ and treated by intraperitoneal injection at the frequency and with the doses described. Labetuzumab govitecan and h679-SN-38 doses were prepared fresh through reconstitution with 0.9% preservative-free sodium chloride (McKesson Medical-Surgical). Cisplatin and etoposide (NIH Developmental Therapeutics Program, RRID:SCR_003057) were prepared and stored at room temperature and 4°C, respectively. Mice were monitored biweekly for tumor growth, weight, and body condition score. A complete response is defined as an undetectable tumor.

Complete blood counts and serum chemistries

Retro-orbital bleeds yielding approximately 200 μ L of blood were performed on mice prior to receiving the first dose at enrollment on day 0, as well as on days 14 and 28 of the study. Blood was collected into green top lithium heparin microcontainers (Becton Dickinson) and tested within 24 hours (Phoenix Labs).

Statistical methods

All data are shown as mean $\pm$ SD. For sample sizes less than 40, normality testing was performed with the D'Agostino-Pearson test. For single comparisons, statistical analyses were performed using a two-sided Student *t* test. For multiple comparisons, statistical analyses were performed using ANOVA with Tukey *post hoc* correction. Data not normally distributed were alternatively analyzed using a two-sided Kruskal-Wallis nonparametric test or Brown-Forsythe and Welch ANOVA with Games-Howell nonparametric *post hoc* correction. For correlation analysis, Pearson correlations or Spearman rank correlations were performed for normal and not normal data, respectively. Best-fit curves were generated with linear regression modeling. Significance was defined as $P \leq 0.05$.

All studies were conducted in accordance with the ethical guidelines expressed in the World Medical Association Declaration of Helsinki.

Data and material availability

Raw and analyzed RNA sequencing (RNA-seq) and ATAC-seq data are available at GEO accession number GSE154576. All other materials will be available upon request and completion of a Material Transfer Agreement.

Results

Enrichment of CEACAM5 protein expression in NEPC

To examine CEACAM5 expression across phenotypic subtypes of advanced prostate cancer, we performed immunofluorescence (IF) staining on a clinically and histologically annotated tissue microarray

(TMA) of lethal mCRPC tissues from 34 patients collected at rapid autopsy through the UW TAN program (25). Two of 34 patient samples were excluded because of poor-quality cores, allowing for the complete analysis of 32 patient tissues. Tissues were classified into four tumor subtypes based on IHC staining for AR, PSA, chromogranin A (ChrA), and synaptophysin (SYP): (i) AR-positive prostate cancer (ARPC: AR⁺ or PSA⁺, ChrA⁻, and SYP⁻); (ii) NEPC (AR⁻ and PSA⁻, ChrA⁺, or SYP⁺); (iii) double-negative prostate cancer (DNPC: AR⁻, PSA⁻, ChrA⁻, and SYP⁻); or (iv) amphicrine prostate cancer (AMPC: AR⁺ or PSA⁺ and ChrA⁺ or SYP⁺). Stromal regions of tissue cores were classified on the basis of morphology (Fig. 1A) and excluded from all analyses to focus on tumor parenchyma. Image analysis revealed that the overall level of CEACAM5 expression was heightened in NEPC based on fluorescence intensity (Fig. 1B) and that NEPC cores contained significantly more CEACAM5⁺ cells ($44\% \pm 39.6\%$; Fig. 1C). Integrated CEACAM5 H-scores (% cells stained $\times$ staining intensity) were substantially higher in NEPC (81 ± 87.5 ; Fig. 1D) compared with other prostate cancer subtypes.

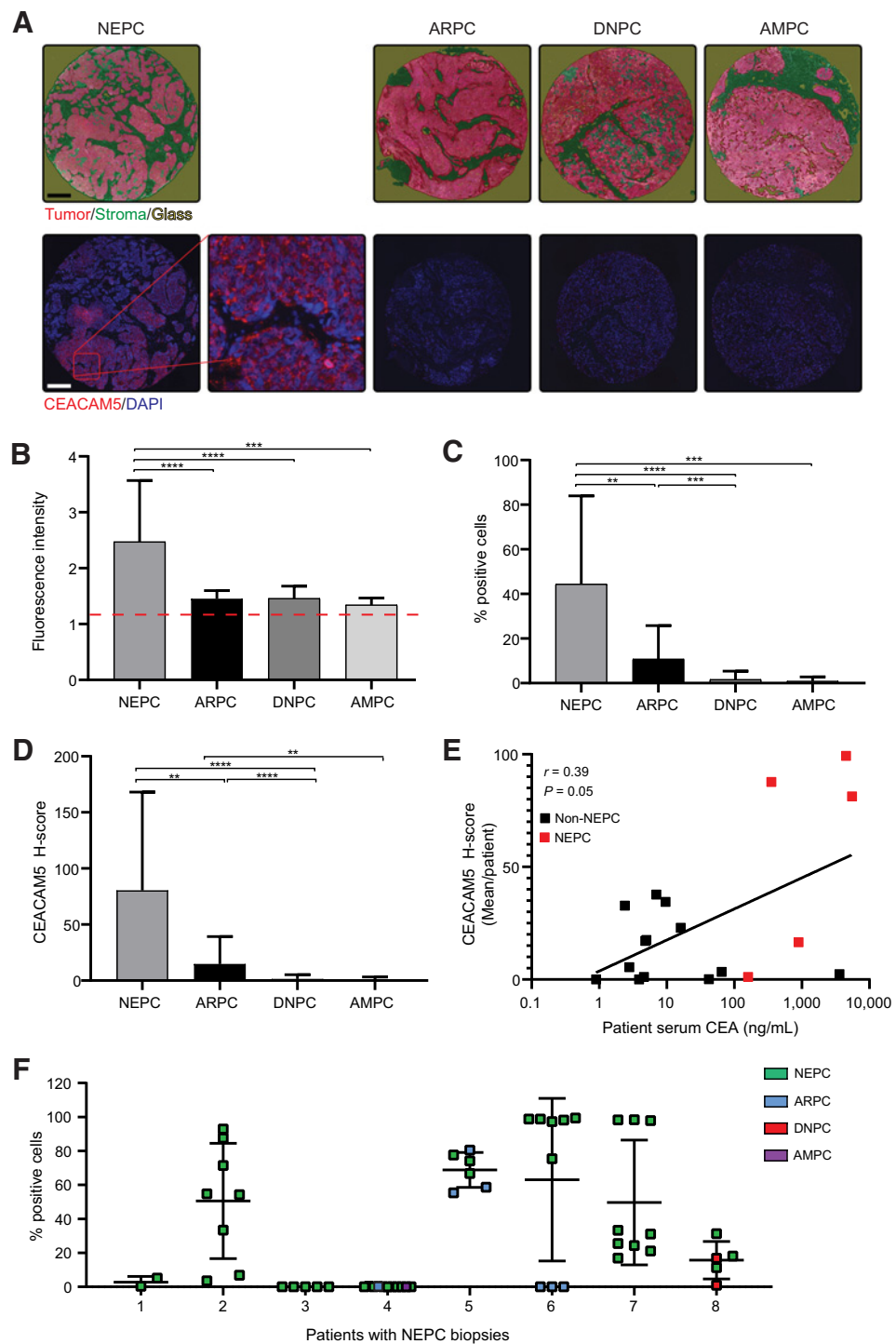
CEACAM5 expressed on the surface of cells is often shed into the bloodstream and can be measured as serum CEA. Serum CEA is a common clinical cancer biomarker but has had a relatively limited role in the clinical management of prostate cancer. Elevation of serum CEA combined with neuroendocrine tumor marker expression has previously been reported as a clinical criterion for aggressive variant prostate cancer, a spectrum of prostate cancers including NEPC that are molecularly characterized by combined defects in *TP53*, *RB1*, and *PTEN* and respond poorly to AR-directed therapies (26). To explore the relationship between serum CEA levels and tumor CEACAM5 expression in lethal mCRPC subtypes, we assayed banked serum samples collected concurrently with tumor tissue from 18 of the 34 patients represented in the UW mCRPC TAN TMA. We found a significant correlation between serum CEA levels and tumor CEACAM5 expression ($r = 0.40$) based on H-score (Fig. 1E). The correlation appeared to be driven primarily by patients with NEPC compared with other mCRPC subsets (Supplementary Fig. S1A and S1B) but subgroup analysis was not statistically significant potentially due to limited sample size. These data suggest that serum CEA could be a valuable adjunct clinical biomarker of NEPC and should be investigated further as a part of prospective clinical trials.

Genomic profiling of prostate cancer by NGS has identified distinct molecular disease subtypes (27). We performed a limited exploratory analysis of whole-exome sequencing of 38 prostate cancer tissues (17 CEACAM5⁺ and 21 CEACAM5⁻) from 28 of 34 patients represented on the UW mCRPC TAN TMA. Our analysis focused on a subset of genes commonly altered in mCRPC including *RB1* and *TP53* and genes in the PI3K/AKT signaling pathway (Supplementary Table S4). Monoallelic or biallelic copy loss of *RB1*, *TP53*, and *PTEN* appeared to be equally common in CEACAM5⁺ and CEACAM5⁻ mCRPC tissues, at frequencies consistent with prior reports (8, 28). Predicted functional mutations were observed in *RB1* and *TP53*, and the mutational frequency was similar in CEACAM5⁺ and CEACAM5⁻ tissues. Monoallelic or biallelic copy loss of *FOXO3*, *MAP3K7*, and *RRAGD* was enriched in CEACAM5⁺ samples compared with CEACAM5⁻ samples by a factor of 2. *MAP3K7* loss has specifically been reported to promote the development of clinically aggressive prostate cancer, and is associated with AR loss and neuroendocrine differentiation (29).

As tissues were collected from multiple metastatic sites (Supplementary Tables S1 and S2) and variable CEACAM5 expression was identified within tissues, we next characterized the inpatient phenotypic heterogeneity of mCRPC in the NEPC samples from the UW

Figure 1.

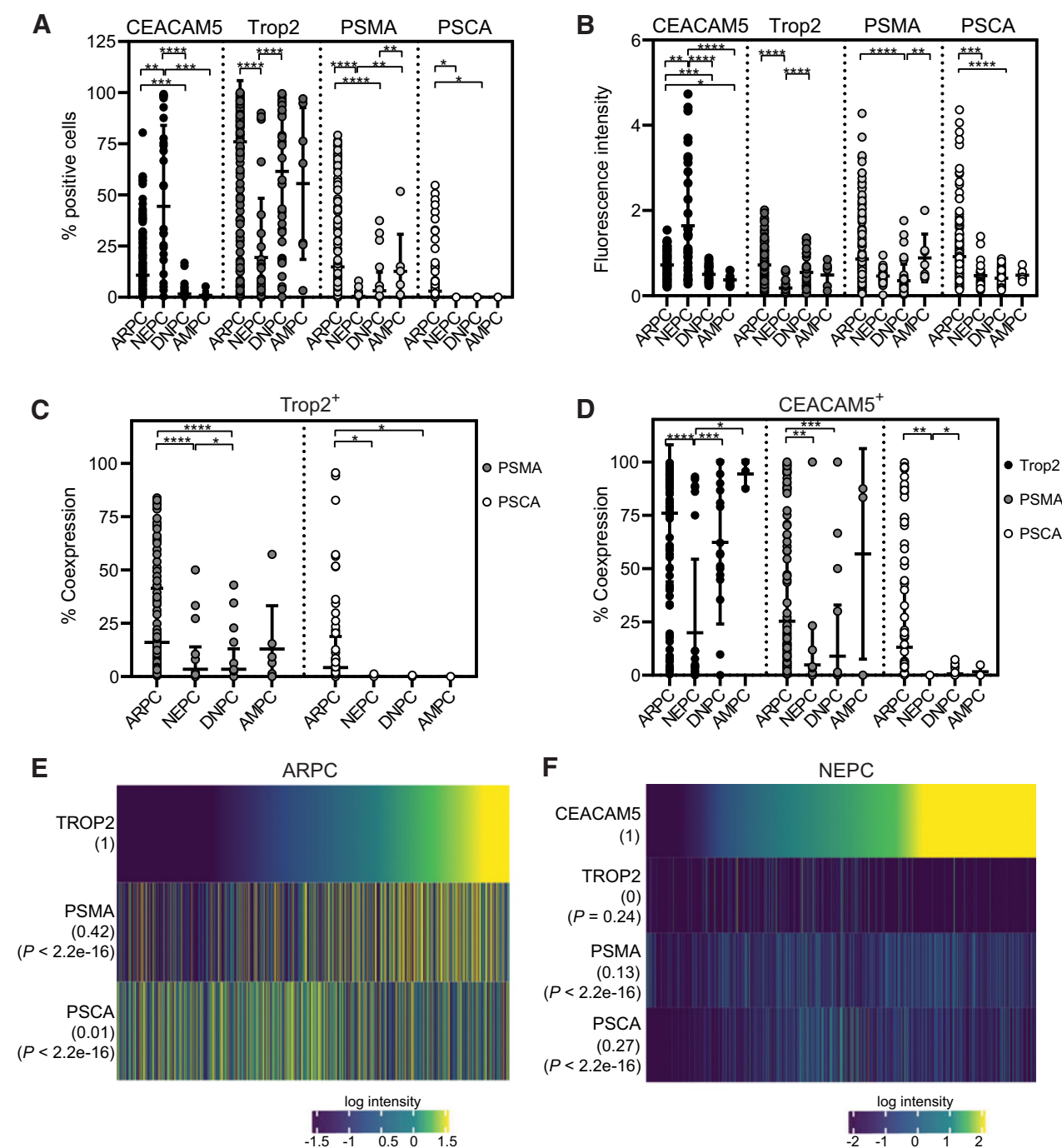
CEACAM5 expression is enriched in the NEPC subtype of mCRPC. **A**, Representative TMA images of individual cores with tumor and stroma annotation as well as fluorescent CEACAM5 (red) and nuclear DAPI (blue) staining (scale bars, 200 μ m; original magnification, 20 $\times$). Intensity of CEACAM5 staining (**B**), percentage of cells with CEACAM5 expression (**C**), and H-scores of neuroendocrine (NEPC, $n = 20$), AR-positive (ARPC, $n = 70$), double-negative (DNPC, $n = 14$), and amphicrine (AMPC, $n = 3$) prostate cancers tissue samples (**D**). **E**, CEA levels in patient with mCRPC serum correlated to relative CEACAM5 protein expression (mIF H-score) in corresponding NEPC ($n = 5$) and non-NEPC (ARPC or DNPC; $n = 13$) patient tumor samples. CEA normal range: 0–5.0 ng/mL. **F**, CEACAM5⁺ cell percentage within the tumor region of cores from all UW mCRPC TAN TMA patient donors with at least one NEPC classified biopsy core. Histograms depict mean $\pm$ SD. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Red Dash = CEACAM5 staining intensity positive threshold. r , correlation coefficient. Kruskal-Wallis values are shown for plots **B**, **C**, and **D**. Spearman rank correlation coefficient (r) and P value is shown for plot **E**.



mCRPC TAN cohort. Four of eight (50%) patients with NEPC had mixed disease based on the presence of additional histologic phenotypes at other tumor sites (**Fig. 1F**). To evaluate CEACAM5 expression in the context of this inpatient heterogeneity, we examined all cores from each of these eight NEPC patients. Five of eight patients (62.5%) were found to have CEACAM5⁺ NEPC (patients 2, 5, 6, 7, and 8). In these five cases, CEACAM5 expression was present at all NEPC tissue sites, albeit with variability in the frequency of CEACAM5⁺ cells

between sites (**Fig. 1F**). In addition, the metastatic samples within these five patients that lacked CEACAM5 expression exhibited non-NEPC phenotypes (**Fig. 1F**). These data further demonstrate enhanced CEACAM5 expression in NEPC, not only across a diverse series of patients, but also within patients harboring phenotypically heterogeneous mCRPC.

We also profiled CEACAM5 expression by IF in a normal human organ TMA (Supplementary Tables S1 and S3). Consistent with prior

**Figure 2.**

CEACAM5 expression relative to other targetable prostate cancer cell surface antigens. Percentage of cells expressing CEACAM5, Trop2, PSMA, and PSMA (**A**) and staining intensity from mIF of ARPC ($n = 70$), NEPC ($n = 20$), DNPC ($n = 14$), and AMPC ($n = 3$) tissue cores (**B**). **C**, Coexpression of PSMA and PSMA in Trop2⁺ cells per core. **D**, Co-expression of Trop2, PSMA, and PSMA in CEACAM5⁺ cells per core. **E**, Quantitative single-cell mIF signal intensities of proteins (rows) in cells from ARPC ($n = 655,676$) and NEPC cores ($n = 113,509$, **F**). Error bars represent $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Kruskal-Wallis P values are shown for plots **A–D**. Pearson correlation coefficient (r) and P values for each measured protein is shown numerically next to heatmap rows.

reports, CEACAM5 expression was detectable at low levels in multiple healthy tissues including the lung, stomach, small intestine, and colon (Supplementary Fig. S2A–S2C; refs. 14, 30, 31). However, the intensity of CEACAM5 staining in normal organs was significantly lower than

in NEPC samples represented in the UW mCRPC TAN TMA (Fig. 1D). This difference in expression could signify a therapeutic window for agents directed at CEACAM5 when applied to NEPC. Collectively, these results provide a comprehensive assessment of

CEACAM5 expression in patients with lethal mCRPC, including NEPC, and in healthy human tissues.

CEACAM5 expression relative to other targetable cell surface antigens in prostate cancer

Multiple clinically relevant prostate cancer antigens including trophoblast cell surface antigen 2 (Trop2), prostate-specific membrane antigen (PSMA), and prostate stem cell antigen (PSCA) are the focus of intense clinical development for mCRPC. The Trop2-directed ADC sacituzumab govitecan (IMMU-132) is currently being evaluated in a phase II study for mCRPC (32). PSMA bispecific T-cell engagers, PSMA radioligand therapies, and PSMA and PSCA chimeric antigen receptor T-cell therapies are also under clinical investigation for mCRPC. We focused on characterizing the coexpression of CEACAM5 and these prostate cancer antigens in lethal mCRPC using a multiplex IF (mIF) staining panel on the UW mCRPC TAN TMA (Supplementary Fig. S3). mIF image analysis demonstrated inverse patterns of (i) CEACAM5 and (ii) Trop2, PSMA, and PSCA staining frequencies and intensities in NEPC and ARPC tissue cores (Fig. 2A and B). Specifically, CEACAM5 expression was enriched in NEPC while Trop2, PSMA, and PSCA expression was heightened in ARPC. Furthermore, PSMA and PSCA were frequently expressed in Trop2⁺ cores in ARPC but not in NEPC, DNPC, or AMPC (Fig. 2C). These results are consistent with the prior characterization of Trop2 as an epithelial marker and the established androgen-regulated nature of PSMA and PSCA expression (33, 34). In contrast, Trop2, PSMA, and PSCA were much less frequently expressed in CEACAM5⁺ cores in NEPC (Fig. 2D).

We evaluated mIF data at a single-cell level across all ARPC and NEPC tissue cores to investigate more granular, digital relationships between (i) Trop2, PSMA, and PSCA coexpression in ARPC and (ii) CEACAM5, Trop2, PSMA, and PSCA coexpression in NEPC. Trop2 and PSMA ($r = 0.42$) but not PSCA ($r = 0.01$) expression were correlated in ARPC cells (Fig. 2E). On the other hand, CEACAM5 did not correlate with Trop2 ($r = 0$) or PSMA ($r = 0.13$) and weakly correlated with PSCA ($r = 0.27$) expression in NEPC cells (Fig. 2F). The variable coexpression of Trop2, PSMA, and/or PSCA indicate the presence of highly heterogeneous ARPC cell populations in lethal mCRPC. Furthermore, these findings suggest that diagnostic and therapeutic modalities under investigation to target Trop2, PSMA, and PSCA in prostate cancer may not effectively localize and treat CEACAM5⁺ NEPC.

Association between ASCL1 and CEACAM5 expression in NEPC

CEACAM5 is highly expressed in colorectal cancer where prior studies have implicated TGF β and retinoic acid signaling in CEACAM5 transcriptional regulation (35, 36). However, little is known about the regulation of CEACAM5 expression in other cancer types including NEPC. On the basis of published literature, we discovered that CEACAM5 is expressed in some neuroendocrine carcinomas such as medullary thyroid carcinoma (MTC) and SCLC but not others like Merkel cell carcinoma (37, 38). MTC arises from parafollicular cells which represent calcitonin-secreting neuroendocrine cells of the thyroid that require ASCL1 for their development (39). In SCLC, CEACAM5 expression is specifically enriched in the ASCL1^{high} subtype over other subtypes including NeuroD1^{high} SCLC (Supplementary Fig. S4A and S4B). In contrast, Merkel cell carcinoma does not express ASCL1 and instead uniformly expresses NeuroD1 (37, 40).

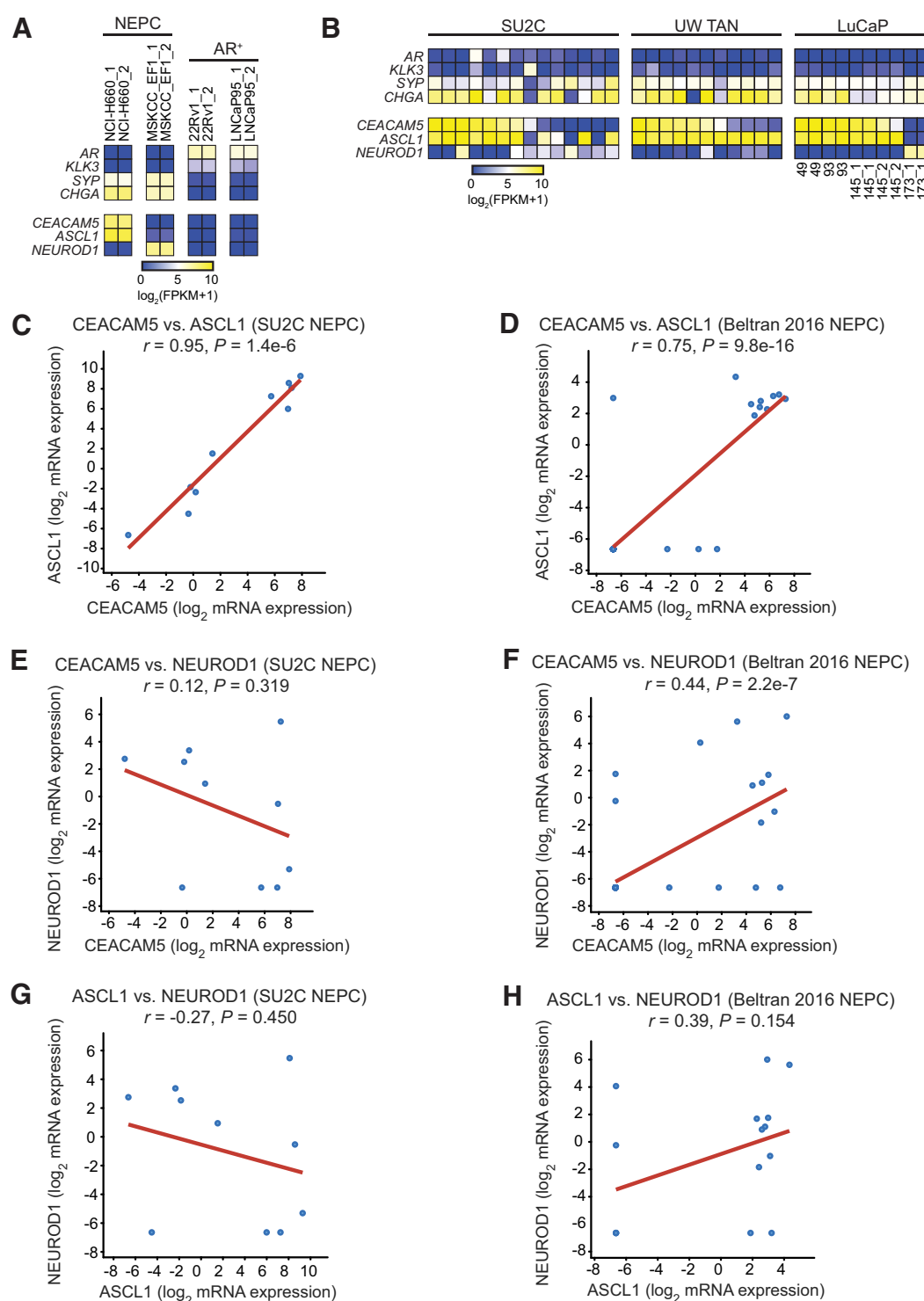
On the basis of these associations in other neuroendocrine carcinomas, we postulated that ASCL1 may regulate CEACAM5 expression in NEPC. To explore this possibility, we first examined the two

available cell line models of NEPC, NCI-H660, and MSKCC EF1. Previously, we have shown that NCI-H660 cells express CEACAM5 and MSKCC EF1 cells do not (14). Transcriptome profiling revealed differential enrichment of ASCL1 in NCI-H660 and NEUROD1 in MSKCC EF1 cells (Fig. 3A), consistent with our hypothesis. We further examined gene expression data from Stand Up To Cancer (SU2C) mCRPC biopsies (41), UW mCRPC TAN rapid autopsies (42), and the LuCaP PDX series (42) to scrutinize CEACAM5, ASCL1, and NEUROD1 expression in NEPC. Across these three datasets, CEACAM5 expression generally associated with ASCL1 expression but not NEUROD1 expression in NEPC samples (Fig. 3B). In the SU2C dataset, CEACAM5 expression was strongly correlated with ASCL1 ($r = 0.95$), but not NEUROD1 ($r = 0.12$) across mCRPC samples demonstrating a neuroendocrine score of >0.4 consistent with NEPC (Fig. 3C and E). The Beltran 2016 NEPC cohort (9) also showed a positive correlation for CEACAM5 and ASCL1 ($r = 0.75$) and interestingly NEUROD1 to a lesser extent ($r = 0.44$; Fig. 3D and F). The correlation between ASCL1 and NEUROD1 expression was negative ($r = -0.27$) in the SU2C dataset while the same comparison showed a positive correlation ($r = 0.39$) in the Beltran dataset (Fig. 3G and H). These findings may reflect increased representation of mixed ASCL1^{high} and NeuroD1^{high} NEPC tumors in the Beltran dataset. Of note, Delta-like 3 (DLL3) is a Notch ligand enriched in NEPC (43) that is the target of multiple therapeutics in clinical development for SCLC and is known to be regulated by ASCL1 (44). CEACAM5 expression correlated with DLL3 expression in the SU2C ($r = 0.54$) and Beltran 2016 NEPC ($r = 0.46$) datasets (Supplementary Fig. S5A and S5B), suggesting that both genes might be regulated by similar programs.

Regulation of CEACAM5 expression during neuroendocrine transdifferentiation of prostate cancer

To uncover possible *cis*-regulatory elements involved in the transcriptional regulation of CEACAM5 in prostate cancer, we examined chromatin accessibility of the CEACAM5 gene locus using ATAC-seq in multiple prostate cancer cell lines, including the NEPC cell lines NCI-H660 and MSKCC EF1 and the AR⁺ cell lines 22Rv1 and LNCaP95. We identified a differential chromatin accessibility peak located at -191 to -92 upstream of the CEACAM5 transcriptional start site encompassing FANTOM5 Cap Analysis of Gene Expression tags of promoter elements in the CEACAM5⁺ NCI-H660 cell line but not in the CEACAM5⁻ MSKCC EF1, 22Rv1, or LNCaP95 cell lines (Fig. 4A). This peak overlaps with the previously described core promoter region spanning -403 to -124 of the CEACAM5 gene locus (45) and was also prominent in pan-cancer The Cancer Genome Atlas ATAC-seq data (46) in tumor types where CEACAM5 is expressed including colorectal (COAD), esophageal (ESCA), gastric (STAD), and breast cancer (BRCA; Fig. 4A). Consistent with these findings, a coinciding DNase I hypersensitivity site was observed in CEACAM5⁺ normal colon tissues but not in CEACAM5⁻ normal breast tissues analyzed by the Encyclopedia of DNA Elements (ENCODE) Project (Supplementary Fig. S6). In addition, the peak heights of the DNase I hypersensitivity site corresponded to reported levels of CEACAM5 expression in colorectal and breast cancer cell lines (Supplementary Fig. S6).

Inferred transcription factor binding from ATAC-seq indicated enhanced activity of ASCL1 in NCI-H660 cells and NeuroD1 in MSKCC EF1 cells (Fig. 4B) which is in concert with their differential expression in these cell lines. However, functional validation studies with short hairpin RNA (shRNA)-mediated knockdown of ASCL1 in NCI-H660 cells and ectopic expression of ASCL1 in MSKCC EF1 cells

**Figure 3.**

Association of ASCL1 and CEACAM5 expression in NEPC. **A**, RNA-seq gene expression heatmap of *ASCL1* and *NEUROD1* in NCI-H660, MSKCC EF1, 22Rv1, and LNCaP95 cell lines. **B**, RNA-seq gene expression heatmap of NEPC samples (columns) from the Stand Up To Cancer (SU2C) mCRPC cohort, the University of Washington Tissue Acquisition Necropsy (UW TAN) lethal mCRPC cohort, and LuCaP patient-derived xenograft lines. **C** and **D**, Correlation dot plots of CEACAM5 and ASCL1, CEACAM5 and NEUROD1 (**E–F**), and ASCL1 and NEUROD1 (**G–H**) gene expression in NEPC samples defined by a neuroendocrine gene signature score >0.4 in the SU2C dataset ($n = 10$) and the Beltran 2016 NEPC dataset ($n = 15$). Pearson correlation coefficients (r) are shown for correlative gene expression analyses.

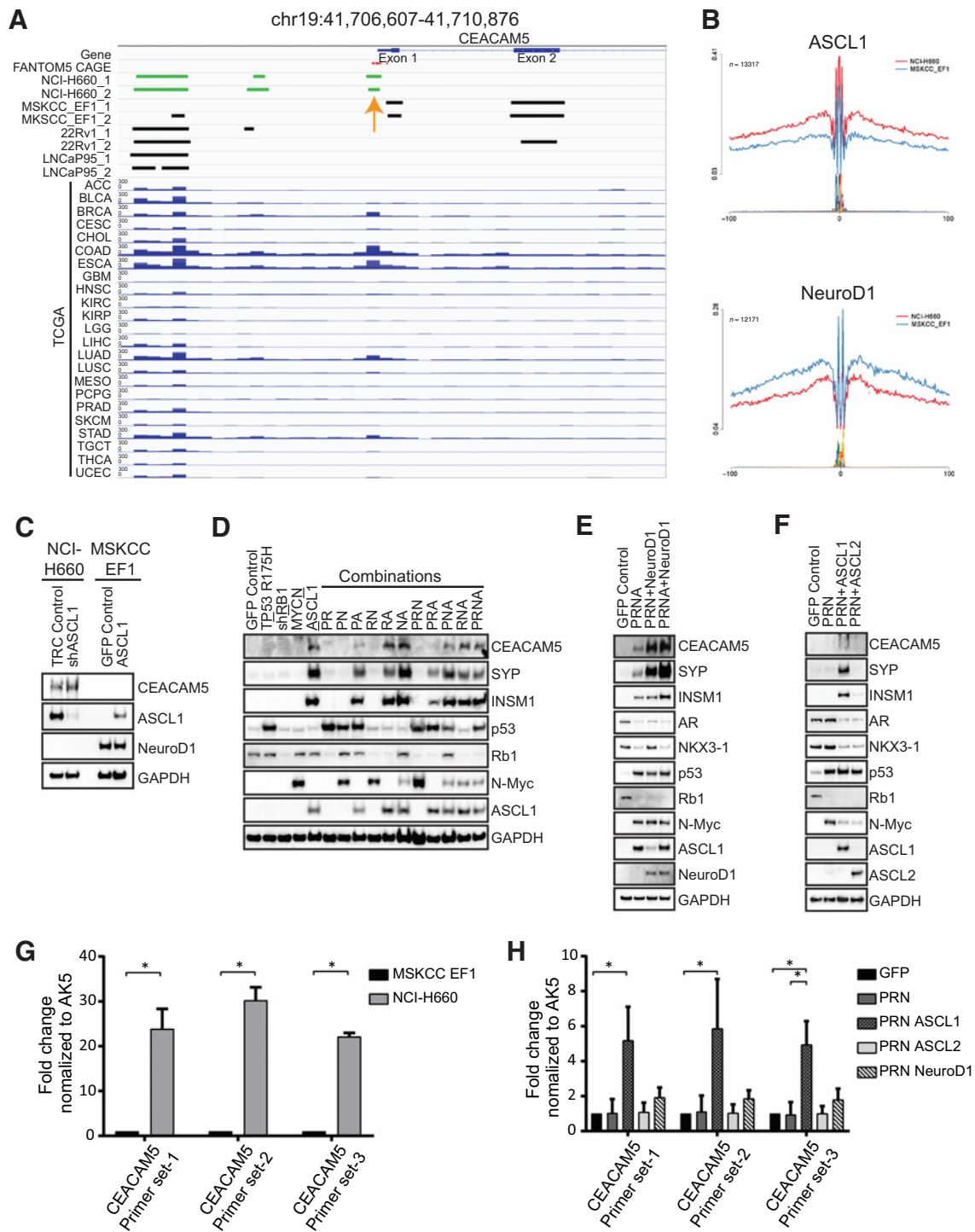


Figure 4.

Regulation of CEACAM5 expression during neuroendocrine transdifferentiation. **A**, Integrative Genomics Viewer tracks showing an ATAC-seq peak at the promoter (orange arrow) upstream of the transcriptional start site of *CEACAM5*. **B**, Lineplots demonstrating inferred ASCL1 and NeuroD1 activity in the NCI-H660 and MSKCC EF1 cell lines using differential transcription factor binding motif footprinting of ATAC-seq data. **C**, Immunoblots demonstrating CEACAM5 protein expression in NCI-H660 cells with ASCL1 knockdown by shRNA and in MSKCC EF1 cells with ectopic ASCL1 expression. **D**, Immunoblots showing CEACAM5 and neuroendocrine differentiation marker expression in C4-2B cells overexpressing ASCL1, NeuroD1 (**E**), or ASCL2 (**F**) in the context of p53 R175H, Rb1 knockdown, and/or overexpression of N-Myc. Chromatin accessibility of the *CEACAM5* promoter determined by ATAC-qPCR in NCI-H660 cells relative to MSKCC EF1 cells (**G**) and C4-2B control cells and cells reprogrammed with ASCL1, ASCL2, or NeuroD1 (**H**). $P = p53$ R175H; $R = shRB1$; $N = N-Myc$; $A = ASCL1$. Histograms depict means $\pm$ SD for biological replicates each with two technical replicates. *, $P < 0.05$. Student t test P values are shown for **G** and Kruskal-Wallis P values are shown for **H**.

had no discernable effect on CEACAM5 expression (Fig. 4C). ASCL1 and NeuroD1 knockdown in the respective NCI-H660 and MSKCC EF1 cells lines was detrimental to cell viability compared with controls (Supplementary Fig. S7A–S7C), indicating perhaps that these lines are genetically hardwired and intolerant of perturbations to these transcription factors. The data could also imply that ASCL1 may not regulate CEACAM5 expression through direct transactivation. To

corroborate this idea, we examined published ASCL1 chromatin immunoprecipitation followed by sequencing (ChIP-seq) data across multiple studies from ASCL1^{high} SCLC cell lines (12, 47, 48), including the NCI-H889 and NCI-H1755 cell lines which express outlier levels of CEACAM5 (Supplementary Fig. S8A). These analyses indicate the absence of ASCL1 binding peaks near the *CEACAM5* gene locus (Supplementary Fig. S8B) but the presence of previously characterized

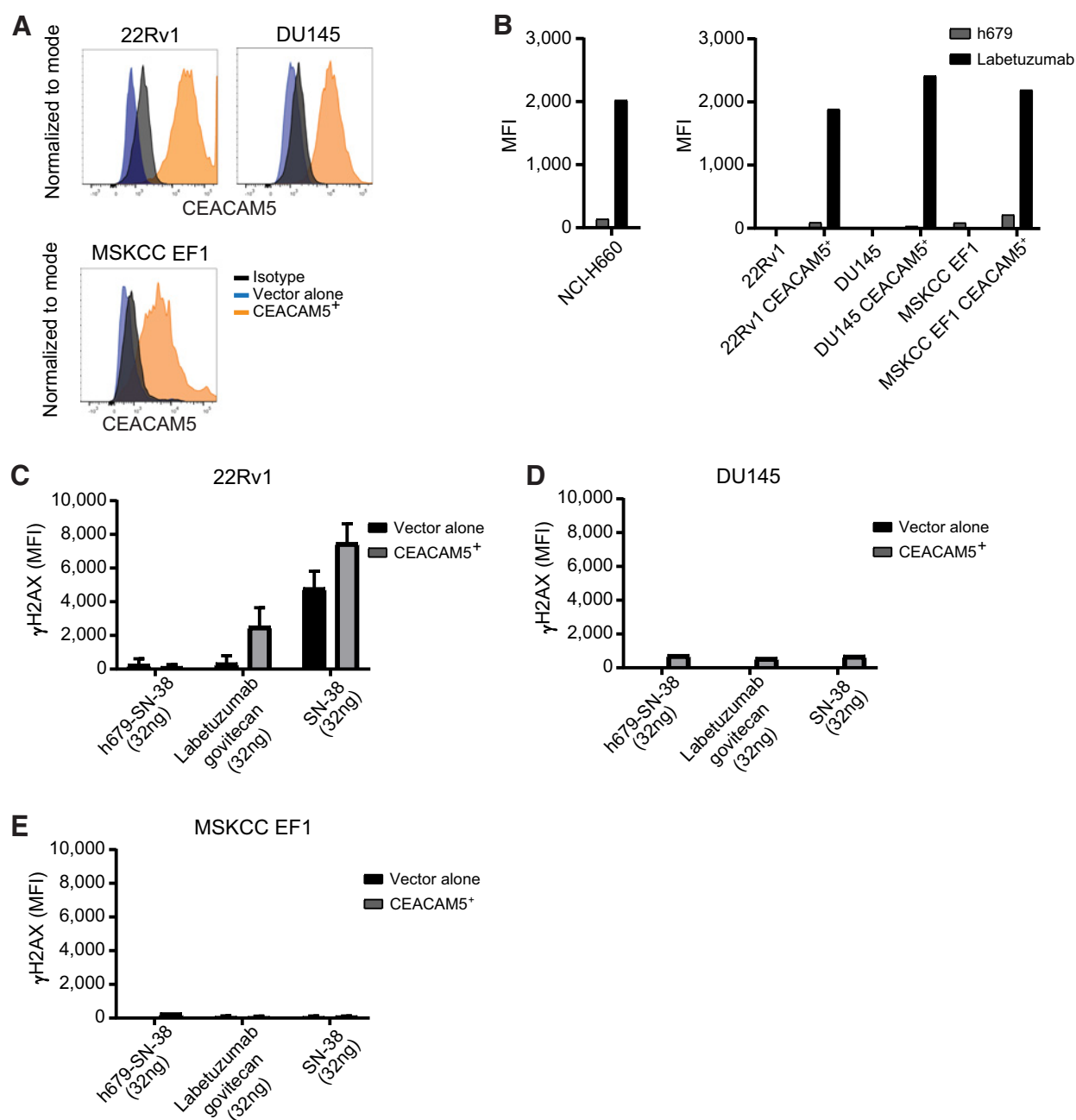


Figure 5.

Labetuzumab govitecan induces dsDNA damage in a CEACAM5-specific manner. **A**, CEACAM5 surface protein expression determined by flow cytometry in prostate cancer cell lines transduced with lentiviral expression constructs. **B**, Labetuzumab binding to CEACAM5 in prostate cancer cell lines. Measurement of intracellular γH2AX staining of 22Rv1 (**C**), DU145 (**D**), and MSKCC EF1 (**E**) cells 16 hours after treatment with h679-SN-38, labetuzumab govitecan, or SN-38 for 30 minutes on ice. MFI, mean fluorescence intensity. Histograms depict means + SD for experimental duplicates.

peaks associated with genes bound by ASCL1 such as *DLL3* and *BCL2* (refs. 12, 44; Supplementary Fig. S8C and S8D). We therefore hypothesized that ASCL1, as a pioneer neural transcription factor, may epigenetically regulate *CEACAM5* by chromatin remodeling. We also reasoned that genetic studies in the hardwired NCI-H660 and MSKCC EF1 NEPC cell lines may not recapitulate dynamic epigenetic regulation of *CEACAM5* expression that occurs during the progression of human prostate cancer.

As an alternative approach, we developed a genetically defined system to induce neuroendocrine transdifferentiation of prostate cancer. We introduced *ASCL1* and other factors causally associated with neuroendocrine transdifferentiation of prostate cancer including dominant-negative *TP53* R175H, shRNA targeting *RB1* (shRb1), and *MYCN* either alone or in combination into the androgen-independent ARPC cell line C4-2B. While C4-2B cells do not express *CEACAM5* at baseline, we discovered that all conditions in which *ASCL1* was introduced stimulated expression of *CEACAM5* and the neuroendocrine markers SYP and insulinoma-associated protein 1 (INSM1; Fig. 4D). In contrast, all other C4-2B conditions in which *ASCL1* was omitted did not exhibit neuroendocrine differentiation (Fig. 4D). We discovered that ectopic expression of NeuroD1 within this system also induced *CEACAM5*, SYP, and INSM1 expression (Fig. 4E). Notably, expression of ASCL1 and/or NeuroD1 down-regulated AR and AR-dependent NK3 homeobox 1 (NKX3-1) expression (Fig. 4E), indicating that these factors may be critical in orchestrating lineage reprogramming from ARPC to NEPC. We also observed that overexpression of NeuroD1-induced ASCL1 expression and the introduction of both ASCL1 and NeuroD1 further enhanced *CEACAM5* expression (Fig. 4E).

We evaluated a second ASCL family member, ASCL2, in the C4-2B cell line to determine whether these effects may be specific to ASCL1. ASCL2 is also a pioneer transcription factor involved in the specification of multiple lineages including trophoblast (49), Th cells (50), and intestinal stem cells (51). Furthermore, ASCL2 expression is associated with the nonneuroendocrine POU2F3^{high} variant subtype of SCLC (52) and is enriched in multiple cancer types where *CEACAM5* is commonly expressed (Supplementary Fig. S9A–S9C). Enforced expression of ASCL2, in combination with *TP53* R175H, shRb1, and *MYCN*, in C4-2B cells suppressed AR and NKX3-1 expression, but did not upregulate *CEACAM5*, SYP, or INSM1 expression (Fig. 4F). These data emphasize the differential competence of pioneer transcription factors to effect neuroendocrine transdifferentiation of prostate cancer and induce *CEACAM5* expression within this system.

To investigate the epigenetic regulation of the core promoter of *CEACAM5* in our C4-2B functional studies, we developed ATAC-qPCR assays incorporating universal normalization control primers targeting *AK5* and three unique primer pairs targeting the differential chromatin accessible and DNase I hypersensitive site we identified in the core promoter of *CEACAM5*. The assays were validated using ATAC libraries generated from the NCI-H660 and MSKCC EF1 cell lines (Fig. 4G). C4-2B cells reprogrammed with ASCL1 revealed a five-fold enhancement in chromatin accessibility at the core promoter of *CEACAM5* relative to control conditions (Fig. 4H). In contrast, no increase in chromatin accessibility was associated with ASCL2 and only a minor, nonsignificant increase was associated with NeuroD1 (Fig. 4H). These results point to one mechanism by which neuroendocrine transdifferentiation driven by ASCL1 may be epigenetically linked to *CEACAM5* expression in prostate cancer.

In vitro specificity and cytotoxicity of labetuzumab govitecan in NEPC

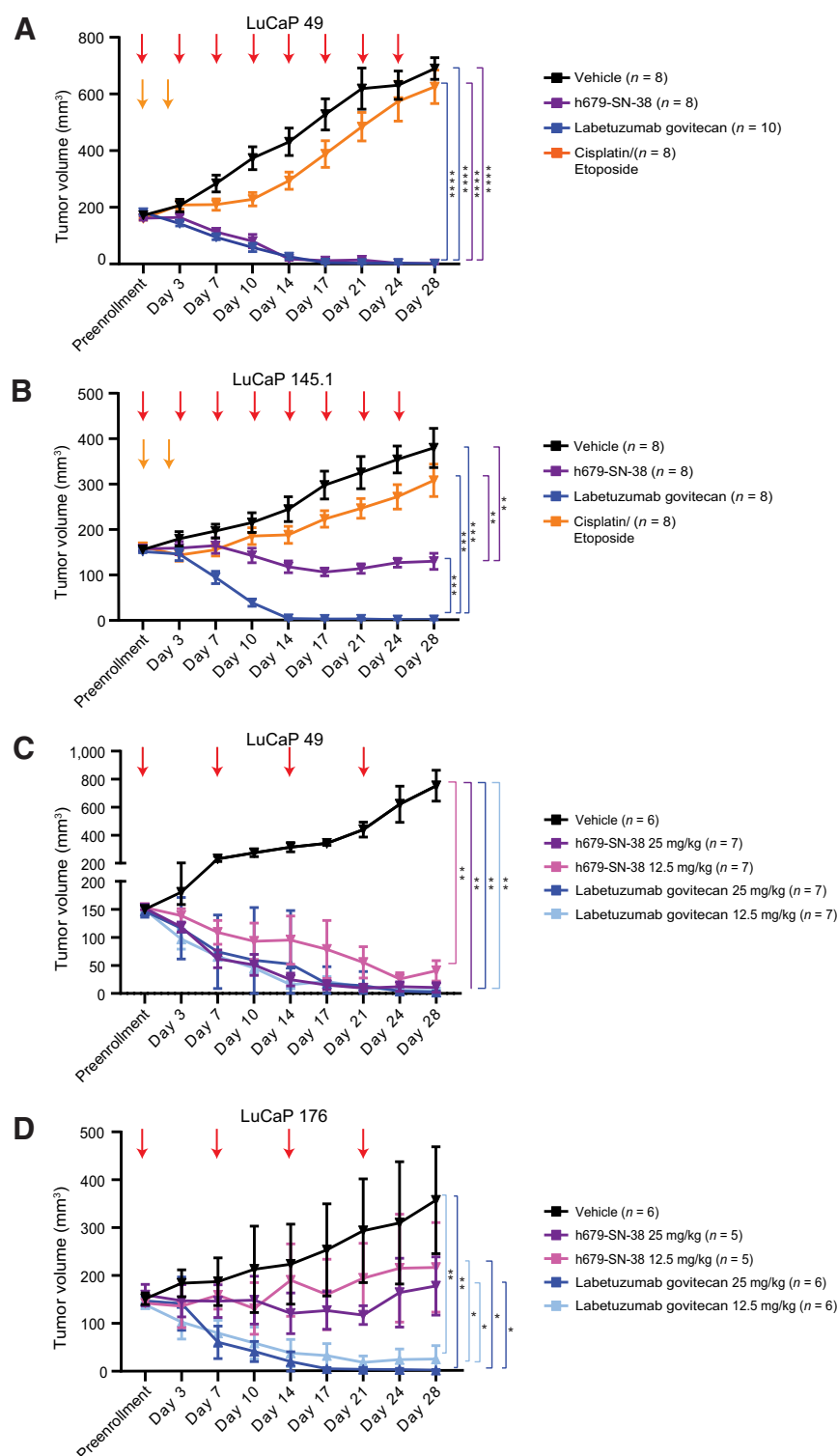
We previously reported that a *CEACAM5* chimeric antigen receptor T-cell therapy demonstrates antitumor activity in NEPC cell line models (14). However, we recognized the lengthy time horizon and numerous hurdles to advancing this type of cancer treatment to the clinic. We therefore concentrated on studies to target *CEACAM5* in prostate cancer by redirecting the established *CEACAM5* ADC labetuzumab govitecan with the anticipation that compelling results could lead to an accelerated path to clinical translation. We first characterized the specific binding of labetuzumab, the humanized antibody component of labetuzumab govitecan, to prostate cancer cell lines with native and engineered expression of *CEACAM5*. *CEACAM5* was stably expressed in three *CEACAM5*⁺ prostate cancer cell lines: the AR⁺ line 22Rv1, the AR[−] line DU145, and the NEPC line MSKCC EF1 (Fig. 5A). We detected labetuzumab binding in all four cell lines expressing *CEACAM5* as well as the natively *CEACAM5*⁺ NCI-H660 cell line, but not in isogenic negative control cell lines (Fig. 5B).

We then investigated the genotoxic effects of labetuzumab govitecan on the prostate cancer cell line panel by measuring γ H2AX, a marker of double-stranded DNA (dsDNA) breaks. Cells were incubated with labetuzumab govitecan for 30 minutes, extensively washed to remove unbound drug, and propagated in cell culture for 16 hours prior to staining and analysis. Labetuzumab govitecan provoked greater γ H2AX signal in the *CEACAM5*⁺ 22Rv1 cell line relative to the control 22Rv1 cell line and compared with incubation with the nonspecific ADC, h679-SN-38 (Fig. 5C). In contrast, SN-38 alone induced γ H2AX in an antigen-independent manner in both the *CEACAM5*⁺ and *CEACAM5*[−] 22Rv1 cell lines (Fig. 5C). h679-SN-38, labetuzumab govitecan, and SN-38 did not generate substantial γ H2AX signal in the DU145 and MSKCC EF1 cell lines, irrespective of *CEACAM5* expression status (Fig. 5D and E). To determine the overall susceptibility of the cell lines to SN-38, we assessed γ H2AX levels following a longer exposure to SN-38 in culture. After a 16-hour incubation, SN-38 induced γ H2AX in all three cell lines (Supplementary Fig. S10A–S10C), albeit to different extents consistent with drug sensitivity based on IC₅₀ calculations from dose–response curves in each of the cell lines with the exception of DU145 (Supplementary Fig. S10D). These data confirm the specificity of labetuzumab binding and the genotoxicity of labetuzumab govitecan in *CEACAM5*⁺ prostate cancer cell lines which generally correlates with the relative sensitivities of the lines to SN-38.

In vivo antitumor activity of labetuzumab govitecan in NEPC

We first examined the antitumor activity of labetuzumab govitecan *in vivo* using *CEACAM5*⁺ NCI-H660 NEPC cell line xenograft tumors established in NOD-scid IL2r^{null} (NSG) mice. Mice were treated with labetuzumab govitecan, h679-SN-38, or vehicle by intraperitoneal injections weekly for a total of four treatments over 28 days. By day 17 and day 24, 100% of tumors in the labetuzumab govitecan treatment arm (*n* = 10) and the h679-SN-38 arm (*n* = 9) were undetectable, respectively (Supplementary Fig. S11A). In contrast, tumors in the vehicle treatment arm demonstrated uncontrolled growth (Supplementary Fig. S11A). No significant changes in mouse weight (Supplementary Fig. S11B) or body condition score (Supplementary Fig. S11C) were observed throughout the study at the 25 mg/kg dose. Four of nine (45%) vehicle-treated mice were sacrificed prior to completion of the study as they exceeded institutional tumor size restrictions (Supplementary Fig. S11D).

We next tested labetuzumab govitecan treatment in multiple LuCaP PDXs established from lethal mCRPC tissues (53) that express varying

**Figure 6.**

Labetuzumab govitecan eradicates CEACAM5⁺ LuCaP PDXs *in vivo*. Tumor volumes monitored biweekly are shown for single-dose trials (**A** and **B**) and two dose trials (**C** and **D**). **A** and **B**, Mice received eight treatments (red arrows) over 28 days with vehicle, h679-SN-38 (25 mg/kg), or labetuzumab govitecan (25 mg/kg). Cisplatin (5 mg/kg) was administered on day 0 and etoposide (8 mg/kg) was administered on days 0 and 2 (orange arrows). **C** and **D**, Mice received four treatments (red arrows) over 28 days with vehicle, h679-SN-38, or labetuzumab govitecan at the doses indicated. Line graphs depict means $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Day 28 ANOVA P values are shown for all panels.

levels of CEACAM5. The LuCaP 49 and LuCaP 145.1 NEPC PDXs were classified as CEACAM5^{low/moderate} and CEACAM5^{high} expression models, respectively, based on intensity of IHC staining (Supplementary Fig. S12). Mice were treated with labetuzumab govitecan or h679-SN-38 at 25 mg/kg or vehicle by intraperitoneal injection every 4 days. Complete responses were observed in 100% of labetuzumab

govitecan ($n = 10$) and h679-SN-38-treated mice ($n = 8$) bearing LuCaP 49 PDX tumors by day 14 (**Fig. 6A**). Complete responses were also observed in 100% of labetuzumab govitecan-treated mice ($n = 8$) with LuCaP 145.1 PDX tumors by day 14, while h679-SN-38 treatment suppressed tumor growth but did not eradicate tumors in any mice (**Fig. 6B**). Importantly, the LuCaP 49 and LuCaP 145.1 tumor models

were relatively resistant to cisplatin and etoposide chemotherapy (Fig. 6A and B) which is considered the standard-of-care front-line treatment for extensive stage NEPC.

In the LuCaP 49 study, the average weight loss in the labetuzumab govitecan group comparing treatment preenrollment with day 28 was 10%. However, this weight loss occurred within the first week of treatment and weights otherwise remained stable in all groups for the remainder of the study (Supplementary Fig. S13A). In addition, no significant changes in body condition scores were observed (Supplementary Fig. S13A). No significant changes in weight or body condition score were observed in mice in the LuCaP 145.1 study (Supplementary Fig. S13B). Adverse effects on liver and kidney function are often reported in association with irinotecan chemotherapy. We performed serum chemistries on days 0, 14, and 28 to assess for these and other toxicities (Supplementary Fig. S14A). Across both studies, three of 18 (17%) labetuzumab govitecan-treated mice exhibited elevated aspartate aminotransferase levels at day 28 that were less than twice the upper limit of the reference range (Supplementary Fig. S14B and S14C), indicating mild hepatotoxicity in these animals. Complete blood counts were also performed (Supplementary Fig. S14D). Across both studies, six of 18 (33%) labetuzumab govitecan-treated mice exhibited leukocytosis at day 28 (Supplementary Fig. S14E) with an increase in the neutrophil fraction (Supplementary Fig. S14F). Similar results were observed in the h679-SN-38 and cisplatin and etoposide-treated mice compared with vehicle-treated mice (Supplementary Fig. S14D).

Given the striking antitumor effects but mild toxicities associated with labetuzumab govitecan at the 25 mg/kg dose, we tested labetuzumab govitecan at a reduced dose with less frequent dosing. NSG mice bearing CEACAM5^{low/moderate} LuCaP 49 NEPC PDX tumors or CEACAM5^{high} LuCaP 176 AR^{low}/NE⁻ PDX tumors were treated with labetuzumab govitecan or h679-SN-38 at 25 mg/kg or 12.5 mg/kg by intraperitoneal injection weekly. In the LuCaP 49 model, both dose levels of labetuzumab govitecan led to complete responses in 100% of mice ($n = 7$) by day 21. While both dose levels of h679-SN-38 inhibited tumor growth, only the 25 mg/kg dose led to tumor eradication (Fig. 6C). The LuCaP 176 model displayed more of a dose-dependent treatment response compared to LuCaP 49. The 25 mg/kg dose of labetuzumab govitecan led to complete responses in 100% of mice ($n = 6$) by day 17. In contrast, tumor eradication was observed in three of six (50%) of mice treated with 12.5 mg/kg of labetuzumab govitecan (Fig. 6D). Both dose levels of h679-SN-38 slowed tumor growth but did not diminish tumor volume. No significant changes in weight or body condition score were detected for either study (Supplementary Fig. S13C and S13D). These studies highlight the potency and efficacy of labetuzumab govitecan in CEACAM5⁺ prostate cancer PDX models by demonstrating that a reduced dose and administration schedule are also capable of achieving complete responses.

Discussion

The development and translation of safe and effective new therapies for NEPC are necessary to alter the course of this highly aggressive and deadly disease. The identification of tumor-restricted cell surface antigens and their targeting with antibodies, ADCs, or adoptive cell therapies has yet to make a clinical impact on the management of NEPC. Recent, substantial efforts have focused on targeting the ASCL1-regulated Notch ligand DLL3, but advanced clinical development of the promising DLL3-targeting ADC rovalpituzumab tesirine was discontinued because of excessive toxicity likely related to the pyrrolbenzodiazepine dimer payload (54). Our work indicates that

CEACAM5 is a compelling cell surface antigen for therapeutic targeting in NEPC as it is expressed in over 60% of NEPC across multiple cohorts of patients, including those with end-stage disease, and demonstrates limited systemic expression. To accelerate therapeutic development, we redirected the existing CEACAM5-targeted ADC, labetuzumab govitecan, currently being evaluated for metastatic colorectal cancer, to NEPC. In multiple preclinical studies, labetuzumab govitecan treatment of patient-derived CEACAM5-expressing tumors resulted in complete responses. Labetuzumab govitecan is similar in design to the ADC sacituzumab govitecan, which was recently approved for the treatment of metastatic triple-negative breast cancer and has received fast-track designation for metastatic urothelial carcinoma and NSCLC. Labetuzumab govitecan and sacituzumab govitecan share the same unique hydrolyzable linker, as well as SN-38 as the cytotoxic payload, and have collectively demonstrated manageable toxicities in patients across several clinical studies (21, 55, 56).

Our studies examining the expression of CEACAM5 and other relevant cell surface antigens in a large cohort of lethal mCRPC samples provide significant biological insights and have important clinical implications. We identified a correlation between serum CEA levels and CEACAM5 expression in tumor tissues across a small series of patients with end-stage mCRPC, which appears most prominent in cases of NEPC. The measurement of serum CEA in the appropriate prostate cancer context (e.g., disease progression with a low PSA) might have value for diagnostic and/or therapeutic purposes in the identification, treatment selection, and disease monitoring of patients with CEACAM5⁺ NEPC. Further investigation of serum CEA as a biomarker in clinical trials for NEPC will be necessary to determine its utility. While expression of Trop2, PSMA, and PSCA has been reported to be relatively homogeneous in early stages of prostate cancer, our results indicate that there is significant heterogeneity in their expression in end-stage mCRPC. Our results show that CEACAM5 expression marks a biologically distinct subset of prostate cancer that has relatively minor overlap with Trop2, PSMA, or PSCA expression. The clinical implication is that CEACAM5⁺ NEPC will not be detected by emerging imaging modalities and may be impervious to treatment approaches directed at Trop2, PSMA, or PSCA.

We also established the functional relevance of ASCL1 and NeuroD1 expression in driving neuroendocrine lineage reprogramming of prostate cancer. These transcription factors appear to induce a simultaneous reduction in AR expression, AR-dependent NKX3-1 expression, and the acquisition of neuroendocrine differentiation markers. Global epigenetic reprogramming of prostate cancer induced by these pioneer transcription factors may coordinately silence the AR-enforced epithelial cancer program and engender neuroendocrine cancer programs. Studies are underway to characterize the contributions of ASCL1 and NeuroD1 to the process of neuroendocrine transdifferentiation of prostate cancer through the integration of genetic, transcriptomic, and epigenetic approaches. Our findings indicate that the biology of NEPC may parallel that of SCLC in that they share ASCL1^{high} and NeuroD1^{high} disease subtypes. However, whether the tuft cell variant POU2F3^{high} or YAP1^{high} subtypes found in SCLC (11) also exist in NEPC has yet to be determined. A recent publication suggests potential biological divergence of NEPC from SCLC in that YAP1 expression is de-enriched in NEPC compared with other subsets of mCRPC (57).

A mechanistic understanding of the regulation of CEACAM5 expression and its specificity to certain cancers has generally been lacking. Previous studies have shown that the wide-ranging modulation of cancer cell differentiation states by retinoic acid or sodium butyrate treatment impacts CEACAM5 expression (36). Our work

demonstrates that ASCL1 promotes neuroendocrine transdifferentiation of prostate cancer which results in increased chromatin accessibility of the core promoter of *CEACAM5*. We suspect that this mechanism of *CEACAM5* regulation by ASCL1 may be conserved in other neuroendocrine carcinomas including SCLC, but additional functional studies will be necessary for confirmation. An interesting question arising from our findings is whether additional pioneer transcription factors may similarly modulate the epigenomes of other tumor types to permit *CEACAM5* expression in nonneuroendocrine cancer cell contexts.

The diversity of prostate cancer phenotypes that emerge with castration resistance and their coexistence in late-stage patients indicate that single-targeted therapies may be ineffective. The existence of multiple subtypes of NEPC that may affect expression of target antigens like *CEACAM5* and *DLL3* in NEPC further compound this issue. Targeted prostate cancer therapies with multiple mechanisms of action or combinations of treatments may be necessary to conquer such diversity. Our *in vivo* studies demonstrate strong antitumor activity of labetuzumab govitecan and, to a lesser extent, the nonspecific h679-SN-38 ADC which is likely a consequence of linker hydrolysis and systemic release of SN-38. Labetuzumab govitecan therefore represents a monotherapy that delivers both regional, antigen-specific and systemic, nonspecific tumor killing. The benefit of a moderately stable ADC linker may be increased efficacy in patients with intertumoral and intratumoral heterogeneity, such as that observed in cases of mixed NEPC which occurs in up to 50% of cases. This bystander effect has also been demonstrated in a number of other tumor types for the sister molecule sacituzumab govitecan (58, 59).

The results of these studies have led to planning for a forthcoming phase I/II clinical trial of labetuzumab govitecan for patients with *CEACAM5*⁺ NEPC. *CEACAM5* is also expressed in other neuroendocrine carcinomas including SCLC and MTC. More than half of SCLC are ASCL1^{high} (11) with the majority expressing *CEACAM5*, while advanced MTC are almost uniformly ASCL1^{high} and express *CEACAM5* (38). Investigation of whether labetuzumab govitecan is effective in these and other *CEACAM5*⁺ neuroendocrine carcinomas may also be warranted.

Authors' Disclosures

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Authors' Contributions

D.C. DeLucia: Conceptualization, data curation, formal analysis, validation, investigation, visualization, methodology, writing-original draft, writing-review and editing. **T.M. Cardillo:** Resources, writing-review and editing. **L. Ang:** Investigation. **M.P. Labrecque:** Resources. **A. Zhang:** Data curation, formal analysis, investigation, methodology. **J.E. Hopkins:** Data curation, formal analysis, investigation. **N. De Sarkar:** Data curation, formal analysis. **I. Coleman:** Data curation, formal analysis. **R.M. Gil da Costa:** Resources. **E. Corey:** Resources. **L.D. True:** Resources. **M.C. Haffner:** Resources. **M.T. Schweizer:** Resources. **C. Morrissey:** Resources. **P.S. Nelson:** Resources, supervision, writing-review and editing. **J.K. Lee:** Conceptualization, data curation, formal analysis, supervision, funding acquisition, investigation, visualization, methodology, writing-original draft, project administration, writing-review and editing.

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