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Prostate cancer research: The next generation; report from the 2019 Coffey-Holden Prostate Cancer Academy Meeting

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Abstract

Introduction: The 2019 Coffey-Holden Prostate Cancer Academy (CHPCA) Meeting, "Prostate Cancer Research: The Next Generation," was held 20 to 23 June, 2019, in Los Angeles, California.

Methods: The CHPCA Meeting is an annual conference held by the Prostate Cancer Foundation, that is uniquely structured to stimulate intense discussion surrounding topics most critical to accelerating prostate cancer research and the discovery of new life-extending treatments for patients. The 7th Annual CHPCA Meeting was attended by 86 investigators and concentrated on

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CONFLICT OF INTERESTS

AS is an employee of The Institute of Cancer Research, which has a commercial interest in abiraterone. AS has received travel support from Roche-Genentech and speaker honorarium from Astellas Pharma. HY is on the advisory board of Johnson & Johnson (Janssen). The authors declare no other conflicts of interest.

many of the most promising new treatment opportunities and next-generation research technologies.

Results: The topics of focus at the meeting included: new treatment strategies and novel agents for targeted therapies and precision medicine, new treatment strategies that may synergize with checkpoint immunotherapy, next-generation technologies that visualize tumor microenvironment (TME) and molecular pathology in situ, multi-omics and tumor heterogeneity using single cells, 3D and TME models, and the role of extracellular vesicles in cancer and their potential as biomarkers.

Discussion: This meeting report provides a comprehensive summary of the talks and discussions held at the 2019 CHPCA Meeting, for the purpose of globally disseminating this knowledge and ultimately accelerating new treatments and diagnostics for patients with prostate cancer.

Keywords

artificial intelligence; cancer immunotherapy; precision medicine; therapeutics; tumor genomics

1 | INTRODUCTION

The Prostate Cancer Foundation (PCF) is a non-profit organization that funds global research focused on advancing the understanding of prostate cancer biology and the development of new treatments for men with aggressive and/or advanced disease. In addition to funding research, the PCF extends its service to the research community and patients by convening two annual research conferences and conducting other programs to accelerate the global exchange of knowledge, and create new research partnerships and initiatives in areas of critical unmet need in prostate cancer research.

The Coffey-Holden Prostate Cancer Academy (CHPCA) Meeting is an annual “think tank” conference, that has been held by PCF for seven consecutive years, and is named in honor of Dr. Stuart Holden and the late Dr. Donald Coffey, two great pioneers of prostate cancer research.[1–6] This conference convenes approximately 75 researchers, with a policy that the organizing committee and at least half of the attendees must be early-career investigators. The CHPCA meeting is designed to maximize discussion by organizing the agenda as short talks followed by long discussion times, in the model of the former NCI Prouts Neck Meetings on prostate cancer.[7] Each year, the meeting addresses highly innovative and critical topics of unmet need that have a significant potential for driving new discoveries and treatment developments in the prostate cancer field.

The 2019 CHPCA Meeting was held at the UCLA Luskin Conference Center in Los Angeles, California, from 20 to 23 June, 2019, and was themed “Prostate Cancer Research: The Next Generation.” The meeting was attended by 86 investigators, including 49 young investigators (57%). The talks and discussions centered on promising new prostate cancer treatment opportunities in preclinical and early clinical stages, issues, and opportunities for optimizing and combining treatments with checkpoint immunotherapy, next-generation technologies, including in genomics, transcriptomics, epigenomics, digital pathology,

artificial intelligence, single-cell technologies, novel multifluidic and 3D methodologies to study tumor biology, tumor heterogeneity and other cells and activities in the tumor microenvironment (TME), and the biology and potential for extracellular vesicles as biomarkers.

2 | DEFINING DRIVERS OF PROSTATE CANCER MOLECULAR AND CLINICAL SUBTYPES

Prostate cancer evolution depends on the acquisition of genomic alterations that drive disease progression. A number of genomic alterations that drive prostate cancer initiation have been identified, including ERG, SPOP, MYC, and androgen receptor (AR) alterations.[8–10] Studies of the molecular landscape of untreated primary prostate cancer have identified distinct molecular subtypes that are defined by underlying molecular aberrations.[9,10] SPOP is an E3 ligase adaptor protein and is commonly mutated in clinically localized and metastatic prostate cancers (overall approximately 10%); and defines a distinct molecular subclass of prostate cancer.[9–11] The biological significance of SPOP mutations in prostate cancer has been studied in detail.[12,13] A conditional mouse model of mutant SPOP (in the setting of PTEN loss) led to the formation of neoplastic lesions confirming SPOP drives prostate tumorigeneses.[12] Mutant SPOP drove increased proliferation and transcriptional signatures consistent with human prostate cancer in mouse prostate organoids.[12] Furthermore, these studies demonstrated that mutant SPOP activates phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) signaling, and upregulates a network of AR associated transcription factors and co-activators.[12] Taken together, mutant SPOP effectively activates two pathways critical to the pathogenesis of prostate cancer.

Critically, a molecular subtype is rarely defined by a single genomic aberration. Consistent with this, deletion of the chromatin remodeler CHD1 commonly co-occurs with SPOP mutations in prostate cancer.[9–11] A recent study has interrogated the functional importance of CHD1 loss in prostate cancer. Loss of CHD1 leads to redistribution of the AR cistrome, driving a unique AR transcriptional signature enriched for pro-oncogenic pathways and tumor formation in the murine prostate.[14] This change in genome-wide AR binding was consistent with the oncogenic AR cistrome observed in prostate cancer samples.[14] Collectively, this study implicates CHD1 as a critical tumor suppressor in prostate cancer through regulation of AR function. Gene expression profiles from radical prostatectomies have also been utilized to clinically define these molecular subtypes.[15] Analyses by Barbieri et al found that men with SPOP-mutated prostate cancer have a better clinical outcome (measured by longer metastasis-free survival) compared with SPOP-mutated tumors that have an additional loss of CHD1, consistent with both genomic and clinical progression (Liu et al, manuscript in preparation). Going forward, it is important that studies of distinct molecular subtypes of prostate cancer interrogate the functional and clinical significance of underlying genomic aberrations to best guide treatment strategies and the development of novel therapies, to improve the outcome of men with lethal prostate cancer.

3 | DRIVERS OF THE BENIGN TO MALIGNANT TRANSITION

While studies have identified a number of alterations that may drive the development of prostate cancer, these alterations alone do not drive or predict tumor aggressiveness. Identifying the genomic alterations that drive the benign to malignant transition in cancer cells is critical, as tumors can develop these at different pathological stages, and having biomarkers to identify which prostate cancers are at high-risk for progression will aid in earlier identification of patients who should receive more aggressive treatments.

Genomic alterations observed at the benign to malignant transition include loss of telomerase activity, telomere erosion, loss of p53, and loss of DNA damage-repair capability, which are associated with the onset of significant chromosomal instability, and select for the amplification of oncogenes and deletion of tumor suppressor genes.[16] Developing methods to identify and understand the biology of these genomic alterations in the setting of multifocal primary prostate tumors is a challenge.

The ability to develop faithful models of disease is critical to advancing the understanding of biology and developing better treatments for prostate cancer. Multiple mouse models as well as diverse human models are needed, due to the complexity and inter- and intra-patient heterogeneity of human prostate cancer.

Studies using genetically engineered mouse models (GEMM) have demonstrated that prostate-specific PTEN-loss results in the development of highly penetrant prostatic intraepithelial neoplasia (PIN) lesions that are poorly progressive, have long latency, and rarely develop into metastatic disease.[17,18] These data have led to the hypothesis that PTEN-deletion may activate a cell cycle checkpoint response that constrains progression. Studies in murine $PTEN^{-/-}$ prostate epithelial cells identified significant activation of the PI3K/AKT, p53, bone morphogenetic protein (BMP), and transforming growth factor β (TGFβ)-SMAD2/3/4 signaling pathways.[19,20] Mice with prostate-specific co-deletion of PTEN and p53 develop prostate cancer that is locally aggressive but not metastatic. However, mice with prostate-specific co-deletion of PTEN and SMAD4 developed rapidly aggressive, lethal prostate cancer, and 100% of mice developed lymph node and/or lung metastases, although no bone metastases were observed.[19] Expression of cellular movement, cell growth, and proliferation genes were significantly higher in $PTEN^{-/-}$ $SMAD4^{-/-}$ prostate cancer compared with $PTEN^{-/-}p53^{-/-}$ prostate cancer cells. Moreover, the expression of PTEN/SMAD4-regulated genes was able to dichotomize prostate cancer patients into those at low vs. high-risk for biochemical recurrence (BCR), whereas a set of PTEN/p53 coregulated genes did not.[19] A four-gene set of direct SMAD4-regulated genes (PTEN, SMAD4, CCND1, and SPP1) was identified that was prognostic for lethal metastatic prostate cancer.[19] The expression of these genes (low PTEN and SMAD4; high CCND1 and SPP1) in prostate biopsy tissue microarray samples outperformed Gleason score in identifying lethal versus indolent cases, while combining the 4-gene PTEN/SMAD4 score with Gleason scoring outperformed either factor alone.[19] Altogether, these data identify the TGFβ-SMAD4 pathway as a major progression barrier in mouse and human prostate cancer and suggest that the expression of SMAD4-regulated genes is prognostic for outcomes. Whether

this pathway serves as a treatment target and how to improve the prognostic potential of the 4-gene PTEN/SMAD4 score deserves further investigation.

Telomere erosion and telomere-based crisis may be a major driver of genomic instability and enables the acquisition of genomic alterations that drive cancer progression. GEMM mice able to model telomere erosion and telomere reactivation have been generated (G3/4 *LSL-mTert)*. When crossed onto the prostate-specific PTEN$^{-/-}$p53$^{-/-}$ background, mice developed prostate cancer with lumbar spine metastases (penetrance of 5/20, 25%).[21] PTEN $^{-/-}$p53$^{-/-}$mTERT tumors also exhibited copy number alterations characteristic of human prostate cancer, including frequent *MYC* amplification and *SMAD4* deletion.[21] Fourteen genes were identified that were commonly altered in human prostate cancer and PTEN $^{-/-}$p53$^{-/-}$mTERT mice (SMAD2, SMAD7, RBL2, DCC, PARD3, ERCC3, MBD2, MTERF, ATP5A1, ATP6V1C1, CyC1, CUL2, PTK2, and SMAD4) and were associated with metastatic disease. Expression of these genes, combined with the 4-gene PTEN/SMAD4 score discussed above were highly predictive for BCR-free survival in two independent prostate cancer datasets and outperformed either gene set alone.[21] These findings demonstrate that appropriate mouse models can be useful for identifying genes and pathways that are clinically relevant in human prostate cancer.

Loss of SMAD4 may also drive the development of an immunesuppressive TME, as PTEN $^{-/-}$SMAD4$^{-/-}$ prostate tumors have increased numbers of infiltrating CD11b^{+} myeloid-derived suppressor cells (MDSCs) and decreased numbers of CD8^{+} T cells, compared with PTEN$^{-/-}$ tumors.[22] Recruitment of MDSCs was found to proceed through YAP1-dependent upregulation of the chemokine CXCL5 in PTEN$^{-/-}$SMAD4$^{-/-}$ prostate tumors, which promoted recruitment of CXCR2-positive MDSCs.[22] Treatment of these mice with a CXCR2-inhibitor significantly decreased the number of intratumoral MDSCs and slowed tumor growth and progression.[22] Targeting MDSCs may be an effective treatment strategy in prostate cancer. In support of this, in prostate cancer GEMM models, checkpoint immunotherapy (anti-CTLA4 + anti-PD1) was highly synergistic in combination with multi-kinase inhibitors that impact MDSC function, such as cabozantinib and BEZ235, but not with dasatinib, which has strong inhibitory effects on T cells but not MDSCs.[23]

Genomic deletion of tumor suppressor genes is a rite of passage for virtually all human cancers. Collateral lethality is a concept in which deletion of tumor suppressor genes can result in collateral deletion of neighboring “housekeeping” genes, but the cancer cells survive though the activities of redundant housekeeping genes. These redundant but essential paralogs may serve as promising cancer-specific therapeutic targets, numerous examples of which are being pursued by academic and pharmaceutical drug developers.[24–26] A similar concept is a form of synthetic lethality, termed synthetic essentiality, in which certain gene(s) that are never deleted in the context of a tumor suppressor gene loss, may be essential functional surrogates of tumor suppressor gene deficiencies and thus ideal therapeutic targets.[27,28] Synthetic essential gene pairs can also be identified by this mutually exclusively deletion pattern in the cancer genome. For instance, CHD1 was identified as a synthetic essential gene in prostate cancer with PTEN deletion.[28] In this study, CHD1 inhibition led to tumor growth suppression in PTEN-deficient but not in PTEN-intact prostate cancer models.[28] In PTEN-intact cells, CHD1 is constantly degraded, however,

upon PTEN-loss CHD1 becomes stabilized and drives a nuclear factor κB (NF-κB)-dependent prostate cancer progression.[28] Studies to validate CHD1 as a therapeutic target and identify optimal combination treatment approaches are ongoing.

4 | UTILIZING PREDICTIVE MEDICINE TO GUIDE TREATMENT FOR MEN WITH CASTRATION RESISTANT PROSTATE CANCER

The development of precise and accurate predictive biomarkers to guide therapy to clinically benefit men with castration-resistant prostate cancer (CRPC) remains an urgent unmet clinical need. Two promising predictive biomarkers under investigation are the constitutively active AR splice variant-7 (AR-V7; 10% to 75% of cases) that associates with decreased sensitivity to endocrine therapy, and DNA repair defects (20% to 25% of cases) that associate with sensitivity to PARP inhibitor therapy.[11,29–36] However, the development of these important predictive biomarkers is not without its challenges. The significance of AR-V7 testing may only be fully realized when it is studied with active AR-V7 targeting therapies, converting AR-V7 from a negative to a positive predictive biomarker.[37,38] Secondly, not all DNA repair defects confer sensitivity to PARP inhibition, with a recent study demonstrating that men harboring ATM mutations experienced inferior outcomes to PARP inhibitor therapy than those harboring BRCA1/2 mutations (discussed in additional detail below). Further studies will be important to define the optimal predictive biomarker suite for PARP inhibitor sensitivity to provide the greatest clinical benefit for men with lethal prostate cancer.[39,40]

5 | ELUCIDATING NOVEL FUNCTIONS OF DNA REPAIR PROTEINS TO SUPPORT NOVEL THERAPEUTIC STRATEGIES IN PROSTATE CANCER

DNA repair alternations are common (20%−25%) in lethal prostate cancer and are significantly enriched when compared with localized prostate cancer.[11,29,40] Clinical studies of PARP inhibitors (including olaparib and rucaparib) have demonstrated single-agent antitumor activity in men with metastatic CRPC (mCRPC).[31,40] The TOPARP-A trial demonstrated eight out of eight (100%) patients with BRCA1/2 alternations responded to olaparib (composite response assessment) with other DNA repair aberrations (such as ATM, PALB2, or FANCA) also being observed in some responding patients.[31] Beyond single-agent PARP inhibition in DNA repair defective prostate cancers, uncovering novel roles of DNA repair proteins in prostate cancer pathogenesis may identify therapeutic strategies to benefit men with lethal prostate cancer.

One potential candidate is DNA dependent protein kinase (DNA-PK) a key DNA repair protein. The AR plays an important role in DNA repair by promoting the resolution of double-strand breaks and resistance to DNA damage.[41] DNA-PK is a key target of AR during DNA damage, regulating AR-mediated DNA repair and cell survival.[41] In addition, DNA-PK functions as a selective modulator of transcriptional networks facilitating cell migration, invasion, and metastasis, and DNA-PK expression is associated with worse outcomes for men with prostate cancer.[42] More recently, pharmacological targeting of DNA-PK has been shown to inhibit prostate cancer cell growth and suppress DNA-PK mediated

transcriptional processes, including novel functions, such as the regulation of epithelial-mesenchymal transition, immune responses, and metabolic processes.[43] Consistent with previous studies, specific targeting of DNA-PK suppressed AR signaling. In contrast, dual targeting of DNA-PK/TORK using CC-115, which is currently being evaluated in clinical studies, upregulated AR signaling and this could be repressed by targeting the AR with enzalutamide.[43] Finally, co-targeting of DNA-PK/TORK and AR has synergistic growth inhibitory effects in prostate cancer models, and this combination is currently being evaluated in men with CRPC. Taken together, these data demonstrate how detailed interrogation of protein function can lead to bench-to-bed discoveries that have the potential to impact the management and treatment of men with lethal prostate cancer.

6 | TROP2 AS A NOVEL THERAPEUTIC TARGET IN CRPC

In the search to define the molecular mechanisms driving prostate cancer progression and develop new therapeutic approaches for patients with CRPC, Trop2 has emerged as a potential therapeutic target. Trop2 is a transmembrane glycoprotein expressed in a subpopulation of murine and human prostate basal cells with stem cell characteristics[44–47] and is overexpressed in human epithelial cancers. New data suggest that Trop2 is highly expressed in CRPC/neuroendocrine prostate cancer (NEPC) and drives the NEPC phenotype. High levels of Trop2 correlate with biochemical recurrence. Overexpression of Trop2 enhances prostate tumor formation, tumor growth, metastasis, and resistance to androgen ablation, while the loss of Trop2 significantly delays tumor formation, tumor growth and prostate cancer metastasis in vivo. Trop2-driven CRPC/NEPC is sensitive to PARP1 inhibition and PARP inhibitors reverse Trop2-driven NEPC features.

These observations lead to novel opportunities for CRPC/NEPC treatment via targeting Trop2 therapeutically using: (a) small molecule inhibitors of Trop2; (b) antibody-mediated targeting; and (c) pharmacological inhibition of Trop2 downstream targets. In particular, monoclonal antibodies against Trop2 have potent anticancer activity in xenograft models of various carcinomas. Sacituzumab govitecan, an antibody-drug conjugate targeting Trop2-positive cells, has yielded durable objective responses in patients with heavily pretreated metastatic triple-negative breast cancer (TNBC).[48] These discoveries have resulted in the recent opening of a clinical trial testing sacituzumab govitecan in patients with mCRPC progressing on abiraterone or enzalutamide (ClinicalTrials.gov Identifier: NCT03725761).

7 | IDENTIFYING DRUGGABLE TARGETS IN NEPC

While androgen deprivation therapy (ADT) remains the backbone for prostate cancer treatment, the use of more potent ADT regimens has led to an increased emergence of resistance mechanisms independent of AR activity. This lethal phenotype adapts to ADT via lineage plasticity and is no longer reliant on AR expression and signaling. These tumors can typically display neuroendocrine features, altered kinase signaling, stem and basal cell gene expression patterns, and characteristic epigenetic alterations.[49–52] Recent preclinical and clinical data have identified key molecular events driving this phenotype including combinatorial loss-of-function of tumor suppressor genes including *PTEN*, *RB1*, and *TP53*.[52,53] Also, distinct epigenome rewiring is indicated by DNA methylation patterns,[52]

global repositioning of histone marks (L. Ellis et al, unpublished; M. Freedman et al, manuscript submitted) and overexpression of epigenetic regulators including LSD1[54] and EZH2.[49,52] Further, inhibition of EZH2 has demonstrated re-sensitization to ADT in preclinical models.[49,55]

Another emerging driver of CRPC/NEPC is the transcription factor POU3F2 (BRN2). BRN2 is a master transcription factor that controls neuronal differentiation during development and is sufficient to drive neuronal differentiation in embryonic stem cells and fibroblasts. It is highly expressed in neuroendocrine or small cell lung cancer (both de novo and treatment-induced). In prostate cancer, AR directly suppresses BRN2 transcription. As a result, BRN2 expression is the highest in NEPC tumors and significantly increased compared with adenocarcinoma.[56] BRN2 is distributed to chromatin sites enriched with stem cell and neuronal-associated binding motifs and regulates neuronal differentiation programs. Thus, BRN2 is sufficient to induce NEPC and is required for enzalutamide-induced NEPC. BRN2 knockdown suppresses NEPC growth in vitro and in vivo. Therefore, targeting BRN2 is a promising strategy to treat or prevent NEPC.

Zoubeidi et al have used X-ray crystallography of BRN2 and virtual compound screening to identify a BRN2 inhibitor. Their lead compound reduced the affinity of BRN2 for DNA and displaced it from chromatin sites that it regularly occupies in NEPC, resulting in suppressed expression of NEPC drivers and markers (EZH2, ASCL1, SOX2, and PEG10), and increased expression of luminal markers. Treatment with the BRN2 inhibitor also suppressed the expression of NEPC markers and the growth of NEPC cells in vivo.[57,58] Collectively, these results provide a promising pathway towards targeting BRN2 and NEPC in the clinic.

8 | EXPLOITING METABOLIC LIABILITIES IN PROSTATE CANCER

The majority of studies exploring novel therapeutic opportunities in prostate cancer focus on the regulation of gene transcription and messenger RNA (mRNA) synthesis via targeting transcription factors and their complexes. However, an emerging and promising field is examining the regulation of mRNA translation and protein synthesis by growth factors, oncogenic pathways, and stress signals (both internal and external), to unveil specific vulnerabilities that can be exploited for cancer treatment. Cancer cells require rapid protein synthesis to meet their needs for high-speed growth, but this addiction also creates a liability as excessive protein synthesis is bioenergetically demanding and can be toxic to the cell via increased flux of misfolded proteins into the endoplasmic reticulum. Therefore, cancer cells need to be able to handle this form of proteotoxic stress.

Prostate cancer GEMM models driven by either PTEN-loss or hyperactivation of MYC exhibit significantly increased global protein synthesis by about 20%, an expected range for such potent drivers of prostate cancer development.[59] Unexpectedly however, significant suppression in global protein synthesis was observed in mice harboring both genetic events, despite the fact that these mice develop more aggressive prostate cancer.[59] The combination of MYC-gain and PTEN-loss was found to trigger the unfolded protein response, a response to cellular stress that suppresses protein synthesis via phosphorylation of the eukaryotic

initiation factor 2-α(P-eIF2α).[59] In support, an inhibitor of P-eIF2α activity, ISRIB[60], was able to target this adaptive mechanism and selectively trigger cytotoxicity in aggressive patient-derived prostate cancer xenograft models.[59]

9 | EXPLOITING THE AR AXIS AS A CELL CYCLE LIABILITY IN CRPC

While AR inhibition has been the backbone of advanced prostate cancer treatment since the original observations of Huggins and Hodges,[61] there is also substantial preclinical and clinical evidence that overactivation of AR can have inhibitory effects on prostate cancer growth.[62–67] A proposed explanation for the latter effect is that, during prostate carcinogenesis, AR gains an oncogenic function in DNA replication licensing during the S-phase and binds to DNA at origins of replication sites as part of the origin of replication complex (ORC), and must be removed via its degradation in late mitosis. As CRPC cells have significantly elevated AR protein levels due to adaptive autoregulation, as part of their adaptation to AR axis inhibition, their exposure to pharmacologic doses of testosterone results in over-stabilized and ORC-bound AR, which inhibits DNA relicensing, resulting in cell death in the subsequent cycle.[62,65] This has led to the hypothesis that men with CRPC may benefit from rapid cycling between polar extremes of supraphysiologic and castrate testosterone levels, a treatment strategy known as bipolar androgen therapy (BAT). Clinical trial data suggest that BAT is a safe therapy that can yield responses in asymptomatic men with mCRPC and also may resensitize CRPC to androgen ablative therapies in patients undergoing rechallenge.[67] Ongoing studies are aiming to define the exact role for BAT in CRPC management and the optimal strategy for sequencing between androgen and anti-androgen therapies to maximize patient benefit. Moreover, it has been demonstrated that supraphysiologic levels of testosterone (SupT) induce DNA double-strand breaks in prostate cancer cells and promote autophagic degradation, which can activate cytoplasmic DNA sensing pathways and downstream innate immune signaling. These findings suggest that BAT engages the immune system to inhibit tumor growth and that combination of BAT with existing immunotherapeutics, including immune checkpoint blockade, may prove beneficial for the treatment of CRPC.[68]

10 | NUCLEAR PORE-RELATED MECHANISMS AS POTENTIAL THERAPEUTIC TARGETS

The development of new therapies targeting AR signaling, such as abiraterone acetate and enzalutamide has led to improved outcomes for patients with mCRPC and de novo metastatic castration-sensitive prostate cancer.[69–76] However, resistance to abiraterone and enzalutamide remains common, with nearly all cases of the advanced disease being fatal. Therefore, as patients develop resistance to AR-targeting therapies earlier in their disease history, there will be an urgent clinical need to develop novel therapeutic strategies that provide further clinical benefit for this emerging patient population.

Nuclear pores are involved in multiple cellular processes including nucleo-cytoplasmic transport, DNA replication, and DNA damage, mitosis and chromosome stability, and gene expression.[77,78] Importantly, nucleoporins from which the nuclear pore complex is derived, are deregulated during prostate cancer disease progression.[79] POM121, a nucleoporin, has

been shown to be critical for prostate cancer aggressiveness. POM121 enhances the importin beta function that promotes nuclear import of key oncogenic (E2F1 and MYC) and prostate-specific (AR and GATA2) transcription factors that drive prostate cancer progression.[79] Consistent with this, genomic abrogation of POM121 reduced AR and AR-V7 nuclear localization, and is associated with decreased AR signaling in treatment-resistant prostate cancer models; this may provide an attractive therapeutic strategy to overcome oncogenic AR-V7 signaling.[79] Furthermore, targeting the POM121-importin beta axis decreased tumor growth, re-sensitized to standard therapy, and improved survival in prostate cancer models.[79] Interestingly, studies have shown a novel role for nucleoporins beyond nuclear pore formation and function, in modulating prostate cancer cell biology through potential direct gene expression regulation (Rodriguez-Bravo et al, unpublished). Taken together, these data suggest that targeting nuclear pore-related mechanisms may have potential as a therapeutic strategy for the treatment of lethal prostate cancer. Further studies will be required to provide a deeper understating of these processes to ensure such strategies are not associated with increased toxicity, and to identify those patients most likely to benefit from these therapeutic approaches.

11 | OPTIMIZING IMMUNOTHERAPY FOR PROSTATE CANCER

Immunotherapy, particularly treatments that target T cell inhibitory molecules (“checkpoints”), such as programmed cell death protein-1 (PD-1) have proven to be an effective treatment for multiple cancer types[80,81] For instance, in metastatic melanoma, median overall survival outcomes for patients receiving nivolumab + ipilimumab have been recently reported as >60.0 months (not reached).[82] While a subset of patients receiving these therapies experience favorable responses, many still experience disease progression, [80,81,83,84] high-lighting the complexity of the tumor immune microenvironment and the heterogeneity of antitumor immune responses. In prostate cancer, checkpoint immunotherapy has yet to be optimized.

12 | MOLECULAR SUBTYPES THAT ARE SENSITIVE TO CHECKPOINT INHIBITORS

One area of intense investigation is the development of predictive biomarkers to identify men with CRPC who will respond to treatment with immunotherapy. Currently, despite evidence of antitumor activity, the overall response rate to pembrolizumab in molecularly unselected CRPC is low.[85,86] A recent study of anti-PD-1/PD-L1 therapy in men with microsatellite instability (MSI)-high/mismatch repair deficient (dMMR) (approximately 3% of cases) prostate cancer demonstrated 6 out of 11 patients (54.5%) achieved ≥50% prostate-specific antigen (PSA) response.[87] A further molecular subgroup of patients with CRPC (approximately 6.9% of cases) with potential to benefit from immunotherapy are those harboring biallelic loss of CDK12, which associates with increased gene fusions, neoantigen burden, T cell infiltration, and early ≥50% PSA responses (two out of four patients; 50%) to anti-PD-1 therapy.[88] More recently, a study of 48 men with CRPC who received pembrolizumab demonstrated 18 out of 48 patients (38%) achieved ≥50% PSA response.[89] Furthermore, 4 out of 48 patients (8%) achieved ≥90% PSA response, and two of these four

patients had mutations in LRP1b, one of whom also had MSH2 loss and was MSI-high and tumor mutational burden-high.[89] These data provide further evidence of clinical benefit from pembrolizumab therapy in men with CRPC and suggest LRP1b mutations may enrich for anti-PD-1 therapy responsiveness. Taken together, although MSI-high/dMMR, CDK12 loss, and LRP1b mutations are relatively uncommon in CRPC, the enrichment for response to immunotherapy is therapeutically meaningful and requires further evaluation.

13 | PROSTATE CANCER LACKS STEMNICHES TO SUPPORT EFFECTIVE ANTITUMOR IMMUNITY

Recent studies have shown that stem-like $CD8^+$ cells are associated with response to checkpoint blockade therapy.[90,91] In renal and prostate cancer, dendritic cells were observed to closely interact with a stem-like population of $CD8^+$ cells, together constructing a stem-niche where antigen-presenting dendritic cells activate the stem-like $CD8^+$ cells and maintain their proliferation (Kissick, et al, manuscript in press). These stem-niches were enriched in highly immune-infiltrated, that is, “hot,” renal tumors compared with “cold” renal tumors, and may play a key role in reinvigorating and expanding $CD8^+$ cells in response to immune checkpoint blockade. However, these niches were extremely rare in prostate cancer, which may explain their non-responsiveness to anti-PD-1/PD-L1 drugs. Determining the cause of the stem-niche-poor environment of prostate cancer and how to facilitate the seeding of stem-niches in prostate cancer could be of critical importance. In contrast to the rapid T cell activation response and lymphocyte trafficking between lymph nodes and target organs that typically occurs quickly after an acute viral infection, differentiated $CD8^+$ T cells migrate to the tumor to seed the stem-niches in a delayed fashion in response to cancer antigens. A provoking hypothesis to be tested is that poor infiltration of vasculatures (blood vessels and/or lymphatic channels) in prostate cancer significantly compromises the delayed migration of dendritic cells and stem-like $CD8^+$ cells from peripheral blood or lymph nodes into the tumors. Current ongoing studies are aiming to increase vasculature infiltration and test its effects in promoting the establishment of stem-niches and immune response in prostate cancer.

14 | THE ROLE OF IMMUNOTHERAPY FOLLOWING OR IN COMBINATION WITH ANTI-ANDROGEN THERAPY

The prostate cancer immune microenvironment is complex, and gene expression patterns of single cells likely harbor important prognostic information. Recent single-cell RNA sequencing (sc-RNA-seq) studies of melanoma,[90] breast cancer,[92,93] and other solid tumors[94–97] have unveiled the remarkable heterogeneity of the tumor-infiltrating immune cell landscape. However, studies of mCRPC have not been adequately performed, due in part to challenges associated with obtaining bone biopsies, the most common site of metastatic disease.[98,99]

Using the 10X platform, CD45-positive cells were analyzed from metastatic lesions of patients with mCRPC who had progressed on enzalutamide and were subsequently treated with pembrolizumab. T cells were the most abundant cell type across all lesions examined

(bone, lymph node, liver) including from both responding and non-responding patients. A more focused analysis of single $CD8^+$ T cell expression profiles revealed a gene signature associated with response to PD-1 blockade in the enzalutamide-resistant setting. Importantly, this signature could be retrospectively validated in an analysis of bulk RNA-seq data. A simplified gene expression signature capable of stratifying patients who could benefit from PD-1 blockade therapy may provide an important biomarker to determine which enzalutamide-resistant patients should move on to immunotherapy treatment.

15 | COMBINATION TREATMENT APPROACHES TO IMPROVE CHECKPOINT IMMUNOTHERAPY

Another route to improving the efficacy of checkpoint immunotherapies is to identify synergistic treatment combinations. Numerous clinical trials investigating inhibition of PD-1 in combination with a second treatment to enhance responsiveness have been undertaken. Beginning with a single combination trial in 2009, there were over 1100 such registered trials in 2017.[100] As more and more therapies are considered for combination treatments, the possible combinations may soon exceed the number of eligible cancer patients.[100] New approaches are therefore needed to determine rational drug combinations with the highest likelihood of exhibiting synergy.

16 | CANCER VACCINES IN COMBINATION WITH CHECKPOINT IMMUNOTHERAPY

Despite prostate cancer being viewed as an immunologically “cold” tumor, there has been a sustained interest in vaccines that activate antitumor T cell responses. In particular, DNA vaccines would ideally be used to activate tumor-specific cytolytic $CD8^+$ T cells as a treatment for early recurrent prostate cancer. In murine studies, engineered CD8 epitopes with increased binding to MHC class I molecules induce the upregulation of PD-1 on antigen-specific $CD8^+$ T cells. The activity of these cells negatively impacted antitumor responses.[101] Interference with the expression or function of PD-1 at the time of T cell activation by vaccine improved antitumor immunity.[102,103] These preclinical findings were translated into a trial for patients with mCRPC, which tested a DNA vaccine encoding prostatic acid phosphatase (PAP) in combination with pembrolizumab. As in the preclinical model, PD-1 blockade, when used at the time of T cell activation by vaccine, led to PSA declines and objective tumor responses.[104] In follow up studies aimed at deciphering mechanisms of resistance in the setting of combination vaccine and PD-1 checkpoint blockade, increased expression of indoleamine 2,3-dioxygenase (IDO) was observed following treatment, and occurred to the greatest extent in patients that failed to mount a clinical response.[105] In addition to PD-1 checkpoint blockade, concurrent inhibition of IDO may therefore further enhance the activity of engineered prostate cancer vaccines.

The PSA-directed vaccine Prostvac has shown promising results in a few phase II trials through provoking PSA-specific immune responses, however Prostvac failed to meet the primary endpoint of an improvement in overall survival in a randomized double-blinded phase III trial in patients with mCRPC.[106–108] To understand this failure and identify

alternate therapeutic strategies to move forward with, samples from patients treated with Prostvac have been further evaluated. A recent study demonstrated that Prostvac increased tumor-infiltrating lymphocytes (TILs) in different tumor compartments in localized prostate cancer in a phase II neoadjuvant trial (NCT02153918) (N = 27). Both non-compartmentalized and compartmentalized analyses were utilized to examine the distribution of immune cells in the post-treatment prostatectomy tissue compared with that in the baseline biopsy tissue. The non-compartmentalized analysis revealed a significant increase in $CD4^+$ TILs but not $CD8^+$ TILs in the post-treatment tumors. Using compartmentalized analysis by separating tumor environment into core of tumor (CT), invasive margin (IM), and benign glands (NL),[109] the study discovered a distinct influx of TILs in different spatial compartments. Prostvac increased $CD4^+$ TILs in IM and NL, not in CT, and increased $CD8^+$ TILs in CT but not in IM or NL. Further analysis of spatial heterogeneity revealed that there was increased peak density and heterogeneity of $CD4^+$ and $CD8^+$ TILs in all three compartments post-treatment, indicating an immune stimulation effect. The study also looked into the changes in regulatory T cells (T_{REG}), activated T cells, and PD-L1 expression post-treatment (Sater et al, unpublished). It is yet unknown if Prostvac changes distributions of immunosuppressive MDSCs or proximity of different T cell subsets in the tumor. The underlying biology and potential therapeutic implications of the spatial-dependent immune modulation by Prostvac also await further investigation.

17 | TARGETING EZH2 TO ENHANCE RESPONSES TO CHECKPOINT IMMUNOTHERAPY

Across many cancers, two factors that seem most strongly associated with response to checkpoint blockade are tumor mutational burden and an inflammatory gene expression profile.[110] In a pan-cancer study of these parameters, prostate cancer was found to have both low tumor mutational burden and an immunologically “cold” gene expression profile.[111] While the prostate cancer tumor mutational burden is relatively low and cannot easily be therapeutically manipulated, recent data have demonstrated that epigenetic therapy can alter gene expression profiles and potentially reverse resistance to checkpoint blockade. In a recent phase II study testing the efficacy of histone deacetylase (HDAC) inhibition via entinostat in combination with pembrolizumab in 53 men (ENCORE 601), an overall response rate of 19% was observed, with a median duration of the response being 12.5 months.[112] These promising clinical data support additional studies in combining epigenetic modulation with immune checkpoint inhibition.

EZH2 is an essential component of the polycomb repressive complex 2 (PRC2), which maintains epigenetic programs important for development and cellular differentiation. It has recently been reported that EZH2-mediated histone H3 lysine 27 tri-methylation represses the production of T helper 1 (T_H1) chemokines in the ovarian TME, which may provide a mechanism for tumor immune evasion.[113] In further support of this hypothesis, levels of PRC2 components, most notably EZH2, were inversely associated with critical T_H1-type chemokines in colon cancer tissue.[114] Using an EZH2 activity score, prostate cancers with low EZH2 activity demonstrate enrichment of an interferon gene signature. Low EZH2 activity was also found to associate with increased PD-L1 mRNA and protein. Therapeutic

inhibition of EZH2 in combination with checkpoint blockade may therefore be a promising approach to the treatment of prostate cancer.[115] In the Hi-MYC mouse model of prostate cancer, chemical inhibition of EZH2 synergized with PD-1 blockade to significantly slow tumor growth. This effect was dependent upon upregulation of tumor PD-L1, and was associated with enhanced $CD4^+$ and $CD8^+$ T cell trafficking, activation of $CD8^+$ T cells, and skewing of the tumor-associated macrophage (TAM) population toward the M1 phenotype.[116] Efforts are now being made to open a clinical trial testing EZH2-inhibitors in combination with anti-PD-1/PD-L1 checkpoint immunotherapy in prostate cancer.

18 | DDR2 AS A POTENTIAL NEW TARGET TO ENHANCE RESPONSES TO CHECKPOINT IMMUNOTHERAPY

In vivo screens of pooled RNA interference (RNAi) knockdown libraries or CRISPR-mediated gene deletion libraries have been used to identify putative new targets to enhance responsiveness to immunotherapy.[117] In one such screen, the collagen receptor discoidin domain receptor tyrosine kinase 2 (DDR2) was identified as a target to enhance responsiveness to anti-PD-1 immunotherapy. In proof-of-principle studies, DDR2 knockdown synergized with anti-PD-1 therapy in isogenic murine models of bladder cancer, breast cancer, colon cancer, sarcoma, and melanoma. Combination treatment of tumor-bearing mice with anti-PD-1 and dasatinib, a tyrosine kinase inhibitor of DDR2, led to enhanced tumor control and in some cases, complete clearance rarely seen with monotherapy. This work provides a scientific rationale for targeting DDR2 in combination with PD-1 inhibitors, and more generally for synergistic genetic screens using in vivo preclinical models. Studies to evaluate the potential for DDR as a treatment target to enhance immunotherapy responses in prostate cancer are needed.

19 | B7–H3 AS A POTENTIAL CHECKPOINT IMMUNOTHERAPY TARGET IN PROSTATE CANCER

Prostate cancer expresses a low level of PD-L1 (B7–H1), which is thought to be the main reason for the limited success of anti-PD-1/PD-L1 in prostate cancer patients. Efforts have been focused on identifying alternative immune checkpoint targets in prostate cancer.

A B7 family molecule, B7–H3, has been found to be commonly expressed in benign prostatic tissue, with increased expression in prostate cancer. Further, its higher expression was associated with worse disease outcomes.[118] A humanized Fc-optimized anti-B7–H3 antibody, enoblituzumab,[119] was evaluated in a phase I trial and demonstrated benefits in tumor reduction in prostate cancer among multiple tumor types.[120] Together these results indicate that B7–H3 is a potential immune-modulating target in prostate cancer. A phase I/II neoadjuvant trial, treating patients with intermediate to high-risk localized prostate cancer with enoblituzumab, recently completed enrollment at Johns Hopkins Hospital (ClinicalTrials.gov Identifier: NCT02923180), with clinical endpoints maturing and correlative data analysis ongoing. Single-plex immunohistochemistry revealed a significant increase of $CD8^+$ TILs but not T_{REGS} in post-treatment tumor tissue. An expanded high-plex study using the Nanostring DSP platform is ongoing to examine 40 protein targets (mainly

immune markers) as well as RNA profiles in treated tumors, using matched untreated tumors as controls. In each tumor, 12 regions of interest (ROIs), including 10 tumor ROIs and two normal ROIs were profiled. Early data revealed a significant upregulation of T cell related immune markers including cytotoxic molecules in treated tumors compared with controls (Shenderov et al, unpublished).

20 | IL-8 AS A NOVEL TREATMENT TARGET IN COMBINATION WITH CHECKPOINT IMMUNOTHERAPY AND ADT

Resistance to ADT and progression to castration-resistant disease remains a major challenge in the clinic. Recent evidence suggests that ADT can augment the response to immune checkpoint blockade.[85,121] In line with this, recent work utilized a Hi-MYC-CaP cell line[122] in vivo transplant model to identify novel vaccination targets that can be induced by ADT. Instead of identifying novel neoantigens, it was observed that a significant shift in the expression of chemokines occurred. The myeloid chemoattractant chemokine, interleukin-8 (IL-8 or CXCL8), has been demonstrated to be upregulated in human prostate cancer and largely mediated by NF-kB (p65) transcriptional activity.[123] Further, a novel AR binding motif was identified in the proximal *IL-8* promoter, such that AR negatively regulates IL-8 gene expression. Thus, ADT increases IL-8 expression in both patient samples and cell lines. Preclinical in vivo modeling convincingly showed that ADT-induced tumor production of IL-8 was associated with increased tumor infiltration of polymorphonuclear MDSCs (PMN-MDSCs). Antagonism of the IL-8 receptor, CXCR2, or genetic knockout of the IL-8 mouse homolog (Cxcl15) demonstrated therapeutic synergy with ADT, decreasing PMN-MDSC tumor infiltration and generating a tumor response to anti-CTLA4 checkpoint blockade.[124] This work has led to the initiation of a Phase Ib/II trial in patients with hormone-sensitive prostate cancer to investigate either nivolumab or nivolumab plus an anti-IL-8 antibody in combination with short-term degarelix (MAGIC-8, NCT03689699). Among numerous primary objectives, investigators will utilize single-cell RNAseq (sc-RNAseq) from patient samples to identify and understand necessary alterations of the prostate TME that underlie therapeutic response.

21 | TARGETING M2-TUMOR-ASSOCIATED MACROPHAGES TO OVERCOME TUMOR IMMUNOSUPPRESSION IN PROSTATE CANCER

Although recent studies of anti-PD-1 therapy with pembrolizumab in unselected patients with CRPC have demonstrated antitumor activity, the overall response rates have been low.[85,86] Despite efforts to identify predictive biomarkers and enrich for patients that respond to checkpoint inhibitors, it is also important to consider other immunotherapeutic strategies that may enhance efficacy and improve clinical benefit for men with lethal prostate cancer.[87–89,125] One potential strategy is to target M2-TAMs, which are consistently being attracted to cancer lesions where they scavenge debris and facilitate the growth and dissemination of the tumor.[126]

Studies of homogeneous human populations of M1- and M2-TAMs have characterized cell surface markers enriched on M2-TAMs that could be utilized as therapeutic targets to

abrogate their function.[127,128] Using solid-phase extraction of N-glycopeptides followed by liquid chromatography-tandem mass spectrometry on homogeneous macrophage populations, CD206 was demonstrated to be enriched on M2-TAMs (compared with M1-TAMs) and N-glycosylated.[127] In addition, infiltration of CD206-positive macrophages increased in a stepwise fashion from normal prostate tissue to primary untreated castration-sensitive disease, to castration-sensitive regional lymph node metastasis, and to metastatic castration-resistant disease.[129] Importantly, a peptide (RP-182) that binds to the fibronectin type II domain of the extracellular portion of CD206 has been shown to decrease the viability of human M2-TAMs, but not M1-TAMs, in vitro (Jaynes, et al., in press). These studies suggest that targeting CD206 has the potential to decrease the viability of immunosuppressive M2-TAMs within the prostate cancer microenvironment, which may provide an attractive method to augment immunotherapeutic responses in lethal prostate cancer.

22 | HYPOXIA DRIVES TUMOR IMMUNE SUPPRESSION AND IMMUNOTHERAPY RESISTANCE

Despite our best efforts to elicit adaptive immune responses, whether it be by checkpoint blockade therapy, either alone or in combination with an orthogonal therapy, epigenetic modification, or vaccination, important metabolic impediments may also need to be addressed.[130] For instance, hypoxia is known to be prevalent in prostate cancer across preclinical models and in patients. Because T cells fail to accumulate in hypoxic niches, recruitment of suppressive stromal cells tend to dominate these microenvironments.[131] The hypoxia-activated prodrug evofosfamide (TH-302) reduces hypoxia in prostate cancer through a tissue-remodeling process. Interestingly, hypoxia reduction with TH-302 sensitizes otherwise resistant murine models of prostate cancer to blockade of CTLA-4 and PD-1. TH-302 treatment reduces MDSC density in prostate tumor tissue by roughly twofold. In this reprogrammed environment, the combination of TH-302 and checkpoint blockade results in enhanced $CD8^+$ T cell proliferation, cytotoxicity, cell survival, and production of effector cytokines.[132] In a phase I study combining evofosfamide and ipilimumab, patients with hormone-refractory metastatic prostate cancer experienced objective clinical responses. This is important, since this patient population has relatively few therapeutic alternatives. These positive data have generated excitement for a phase II trial combining evofosfamide with dual CTLA-4 and PD-1 blockade, which is planned for enrollment beginning in 2020 at MD Anderson Cancer Center.

23 | STROMAL REGULATION OF PROSTATE CANCER DEVELOPMENT

The role of nonimmune stromal cells, such as myofibroblasts or fibroblasts within the prostate tumor microenvironment is a critical question. Prostate cancer initiation and progression have been found to involve extensive interaction with surrounding myofibroblasts or fibroblasts that undergo histopathological changes during disease advancement. It was demonstrated that in vivo co-implantation of non-tumorigenic human prostate epithelial (BPH-1) cells with human prostatic carcinoma-associated fibroblasts (CAFs) resulted in the malignant transformation of BPH-1 cells. This work highlighted the

potential of stromal fibroblasts to promote prostate cancer initiation and progression.[133] In a recent study, laser capture microdissection on human prostatectomy samples was performed to capture and profile gene expression exclusively from epithelial cells or stroma cells. Interestingly, it was a 29-gene signature from the stromal compartment that predicted the aggressiveness and metastatic potential of these prostatectomy samples.[134] Utilizing a TMPRSS2:ERG-knock-in prostate cancer GEMM model, it was noted that the epithelial compartment exhibited minor changes, whereas significant alterations of stroma adjacent to the epithelial compartment were observed (Pakula et al, unpublished). Use of single-cell RNA-seq identified enrichment of Wnt signaling genes within the prostatic stroma (Pakula et al, unpublished). As this work matures, it will be important to elucidate the extent of Wnt signaling enrichment in stromal cells and its specific role in driving therapeutic resistance and/or metastatic progression. Moreover, identification and validation of therapeutic targets incorporated with the use of a stromal signature may offer patient selection to receive specific stromal-targeted therapies.

24 | NEXT GENERATION TISSUE-BASED ASSAYS TO VISUALIZE TUMOR MOLECULAR PATHOLOGY IN SITU

Various next-generation tissue-based assays are now available for investigators to study novel biomarker targets or to measure large panels of antibody and nucleic acid probes simultaneously without losing information on spatial relationships. These tools enable us to investigate interactions among different molecular targets or among different cell populations in vivo. Studies using these assays have advanced our understanding of the prostate cancer-specific TME and illustrated therapeutic modulation of the immune microenvironment as exemplified by several studies discussed above. In addition, artificial intelligence has taken a promising step forward, demonstrating a trained computational image analysis algorithm is able to predict disease outcome using plain hematoxylin and eosin (H&E) slides of primary prostate cancer.

Digital image analysis and machine learning can discover hidden microscopic morphological features that are associated with disease aggressiveness. In prostate cancer, neuroendocrine differentiation (NED), and chromosomal instability (CIN) of tumor cells are both associated with an aggressive disease phenotype. A study assessed the ability of a computer algorithm that captures morphological features related to NED and CIN in predicting disease outcomes, using a cohort of high-grade prostate cancers from biopsy tissue of M1 (de novo metastasis; N = 150) and M0 (stable disease 5 years after treatment; N = 102) patients. First, a CIN-feature computational model was built and shown to predict disease outcomes with a similar performance as the classic CIN70 RNA-signature.[135] Next, an algorithm that distinguishes small cell-neuroendocrine and adenocarcinoma based on morphometric features was trained. The algorithm provides a SC-NED score, which is the likelihood of a cancer being small cell carcinoma. Combining SC-NED score, CIN-features, serum PSA, and age, the 4-variable model was able to predict metastasis and prostate cancer-specific survival with an area under the curve (AUC) of 0.9. Interestingly, gene signatures correlative to the SC-NED score included genes involved in radiation and docetaxel-resistance, discohesiveness, proliferation, and stemness. Further, the expression of

a gene signature derived from the SC-NED score significantly increased from low to high-grade primary to metastatic prostate cancer (Knudsen et al, unpublished). The study is a convincing example that machine learning will play an integral role in assisting with the assessment of aggressive behavior of prostate cancer in core needle biopsies at the time of diagnosis. In addition, image analysis can extract hidden information from H&E slides that can be combined with genetic analysis to improve personalized patient care.

There is an urgent need for novel assays that go beyond the detection of protein and mRNA expression and allow investigations of epigenetic alterations, protein-protein interactions, and protein-DNA interactions in clinical tumor samples in situ. To this end, two novel assay platforms were developed that allow the detection of DNA repair alterations and site-specific DNA methylation alterations in situ. Both assays are based on the principles of rolling circle amplification (RCA) and the proximity ligation assay (PLA).[136,137] Theses assays work in formalin-fixed paraffin-embedded material and rely on the amplification of a circular DNA probe by isothermal amplification to generate a DNA nanoball that can be visualized directly in the tissue. The first assay, termed in situ DNA damage response (IDDR) assay, relies on the combination of two assay components, PLA and the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The TUNEL assay allows for unbiased labeling of DNA breaks using the enzyme terminal transferase. PLA uses isothermal RCA to detect two interacting targets that are in close spatial proximity (<100 nm). The combination of these two assay principles in the IDDR assay allows for the detection of binding of any DNA repair protein to sites of DNA damage in tissue and is currently being evaluated in two PARP inhibitor trials for its predictive value for treatment response (Haffner et al, unpublished). The second assay is an in situ assay to label sequence-specific DNA methylation. Cancer-specific DNA methylation are useful markers for prostate cancer diagnosis (eg, *GSTP1*)[138] and can be used as heritable markers to study cancer clonal evolution and clonal relationships.[139] This assay includes on-slide bisulfite conversion as the first step, coupled with RCA to generate signals that can be scored on a conventional light microscope. Both in situ assays can be multiplexed, therefore enabling the evaluation of a panel of specific biomarker targets simultaneously (Haffner et al, unpublished).

25 | NOVEL MOLECULAR AND FUNCTIONAL ANALYSES OF SINGLE CELLS FROM THE TME

In an effort to understand how the tumor microenvironment contributes to drug resistance, a multi-disciplinary team at the University of Wisconsin is utilizing PSMA PET/MRI scans to study distinct lesions of multifocal primary prostate cancer. Patients in this study underwent a baseline PSMA PET/MRI scan, followed by three cycles of neoadjuvant chemo-hormonal therapy, a second PSMA PET/MRI, and then prostatectomy. A patient-specific 3D prostate mold was used to perform an image-guided dissection on the prostatectomy specimen and extract distinct tumor foci that appeared sensitive versus resistant to therapy. Evaluation of these distinct tumor foci found increased M2 macrophage infiltration and Trop-2 expression in resistant lesions with concordant decreases in lymphocyte infiltration and MHC class 1 expression on tumor cells (Lang et al, unpublished).

To investigate how these multi-compartment alterations in tumor and immune cells may promote therapeutic resistance, a microfluidic coculture platform that enables testing of up to six different cell types in the same microenvironment, termed STACKS, was developed.[140] STACKS allows for the "stacking" of layers that contain preconditioned microenvironments with three-dimensional tissue models (eg, cancer). This technology allows the investigation of cell-cell interactions, paracrine-signaling events, and analysis of cell behavior including migration and invasion. Because this system requires very few cells, this technique is applicable for use with human samples including patient lymphocytes, monocytes, and tumor organoids. Initial proof-of-concept experiments utilizing prostate cancer cell lines, LNCaP and C4–2B, cocultured with human primary monocytes indicated a differential of monocyte polarization based on gene expression and overall migration towards tumor cells. Additional experiments using DU145 prostate cancer cell lines stacked with patient T cells further demonstrated the ability to track the migration of T cells towards tumor cells at single-cell resolution using confocal microscopy. Additional stacking with patient-matched M2 macrophages inhibited the migration of T cells, indicating the potential for functional assessment of a patient's tumor-microenvironment interaction (Lang et al, unpublished).[140–142]

Pushing 3D culture systems to the next level, Lang et al have now begun to utilize exclusive and/or finite liquid repellency (ELR and FLR, respectively)[143–145] platforms to investigate tumor cell interactions with immune cells at the single-cell level. ELR/FLR enables the culture of single cells in microbubbles submerged in oil. These platforms permit the fluid exchange of media and free gas exchange while eliminating evaporation, thus allowing cultures down to the single-cell level. To date, proof-of-concept experiments using ELR have demonstrated successful single-cell culture of autologous human monocytes that have demonstrated differential potentials of individual monocytes to proliferate or not.[145] Further, the application of FLR allowed for successful single-cell culturing of human C4–2B prostate cancer cells, which demonstrated contrasting abilities of individual cells to proliferate, indicated by differential gene expression profiles.[144,145] ELR-based coculture experiments of human tumor-associated neutrophils with C4–2B tumoroids demonstrated heterogeneous interactions between tumoroid and neutrophil populations, including induction of tumor invasion versus apoptosis.[144,145] These studies and technologies will improve our ability to observe and understand exact tumor-microenvironment interactions and tumor potential for metastatic progression and/or response to therapy.

Using these state-of-the-art and innovative technologies, it is anticipated that the re-creation of a prostate tumor microenvironment using STACKS, ELR, and FLR is feasible, using patient-derived tumor organoids and autologous immune cell populations and stromal cells. Overall, these platforms will allow for critical questions to be addressed including determining: (a) the absolute number of MDSCs or M2 macrophages required to drive immune evasion, (b) the properties of T cells that are able to migrate into a hostile environment, (c) the effects of tumor cell genomics on the tumor microenvironment (including downregulation of HLA expression), and (d) discovering therapies that can produce a tumor microenvironment that is conducive towards response to immunotherapy or eradication of cancer cells.

26 | MASSIVELY MULTIPLEX SINGLE-CELL CHEMICAL TRANSCRIPTOMICS

As next-generation sequencing technologies advance, the potential to multiplex molecular readouts per cell is a reality. Single-cell combinatorial indexing ("sci") is a novel approach for profiling the genomes of thousands to millions of cells without ever resorting to single-cell isolation.[146] Sci-sequencing relies on split-pool DNA barcoding of cells or nuclei by employing various molecular techniques (eg, transposition, reverse transcription, ligation) to incorporate unique DNA barcodes into populations of intact cells/nuclei in separate reactions, after which they are pooled, redistributed, and then subjected to subsequent rounds of molecular barcoding.[146] The unique "journey" taken by every single-cell in the assay through multiple barcoding reactions allows for the linking of sequenced molecules back to cells-of-origin by analyzing the unique combinations of molecular barcodes ("combinatorial indices") associated with each sequencing read. This strategy was used to demonstrate proof-of-concept for high-throughput single-cell sequencing of chromatin accessibility (sci-ATAC), chromosome structure (sci-Hi-C), mRNA expression (sci-RNA), and, most recently, co-measurement of multiple genomic phenomena in the same single cells (sci-CAR).[147–149] Importantly, the split-pool nature of these protocols enables i) processing of very large numbers of cells in single experiments, and ii) single-cell data collection without ever resorting to single-cell isolation (ie, in wells, droplets, etc.).[147–149]

The most recent addition to the sci-family of assays is sci-Chem, a platform pairing chemical screening with sci-RNA-seq (Ramani et al,[149] manuscript in revision]. The assay relies on labeling populations of chemically treated cells with a short single-stranded DNA oligo that is read out during the sci-RNA-seq protocol and allows profiling of approximately 10^5 single-cell transcriptomes after approximately 10^3 different chemical perturbations in a single experimental workflow. A major finding from this pilot screen (approximately 600 000 cells sequenced) was a unified mechanism-of-action for 17 different HDAC inhibitors (HDACi). Across three independent cell lines tested, HDACi resulted in cellular upregulation of nontarget genes that serve as lysine deacetylases (ie, sirtuins), and upregulation of metabolic enzymes required to regenerate acetyl-CoA from non-histone cellular substrates. These data are consistent with a model where HDACi does not act via chromatin remodeling but instead serves to starve cells of an essential intermediate metabolite (acetyl-CoA).

27 | MEASURING CHROMATIN ARCHITECTURE OF NEPC AT THE SINGLE-CELL LEVEL

A new approach to analyzing chromatin architecture apart from histone ChIP-sequencing is assay for transposase-accessible chromatin (ATACseq), which assesses genome-wide chromatin accessibility.[150] Recent advancements have now allowed for ATACseq to be performed at the single-cell level (scATACseq).[151] A proof-of-concept study (Brown et al, unpublished) performed comparative bulk ATACseq and scATACseq analysis utilizing human adenocarcinoma and neuroendocrine prostate cancers from the LUCaP patient-derived xenograft library.[152] Bulk ATACseq analysis revealed key features known to NEPC

including biallelic loss of *RB1*, and motif enrichment analysis indicated loss of accessible chromatin at the *AR* gene locus and gain of an important neuronal transcription factor upregulated in neuroendocrine prostate cancer, ASCL1. These data overlay significantly with transcriptional data from preclinical and clinical samples, involving loss of AR and concurrent gain of ASCL1 transcript.[49,52] Further, unique differences were observed when bulk ATACseq data was applied to principle component analysis, indicating that chromatin accessibility information can confidently provide information pertaining to tumor cell identity. The application of scATACseq also revealed that NEPC have a distinct chromatin accessibility pattern when compared with adenocarcinoma. Motif enrichment analysis from scATACseq data more distinctly revealed the discrepancy by which adenocarcinomas may acquire important transcription factors that drive NEPC cell identity, including SOX2 and ASCL1. Excitingly, this proof-of-concept study was performed from frozen tumor tissue and infers that scATACseq will be a robust assay that can be performed using patient samples to understand the state of prostate cancer progression from adenocarcinoma to NEPC.

28 | NOVEL MECHANISMS TO EDIT GENOMES

Understanding the biological and therapeutic consequences of mutations that promote prostate cancer or arise during therapy requires models that recapitulate these mutations. While biologists can routinely affect the activity of a gene in cell culture systems using tools such as expression plasmids and RNAi, until recently it has been difficult and costly to make durable changes to a cell's genome without otherwise perturbing the cell. Modifying the genome directly allows investigators to evaluate the effects of specific mutations on protein expression, structure, and function under physiologically relevant conditions. The discovery of CRISPR-Cas9[153,154] and subsequent development of protocols employing CRISPR-Cas9 to edit a cell's genome have simplified this task.[155–157] Genome editing with CRISPR-Cas9 targets the Cas9 endonuclease to generate a DNA break near the edit location. Template DNA containing a novel sequence is also introduced into the cell, in hopes that DNA repair by homologous recombination will employ the new DNA as a template to repair the break. However, these methods are typically inefficient, cell type-specific, and often result in stochastic mixtures of genome editing byproducts, especially when introducing point mutations.

Base editing technology is a newer approach to genome editing that does not require double-strand breaks, which can negatively affect cell viability.[158] Base editing couples Cas9 to engineered versions of naturally occurring enzymes, such as cytidine deaminase or adenosine deaminase that mutate C-G DNA dinucleotide pairs to T-A[159] or A-T pairs to G-C.[160] The Cas9 proteins used in base editing are modified so they cannot break both DNA strands. Instead, when Cas9 binds to DNA, it exposes single-stranded DNA within a narrow window to the bound deaminase protein, efficiently producing the desired mutations. Although the ABE7.10 adenosine editor system is a specific DNA editor, it generates off-target RNA edits.[161] The third generation BE3 cytosine base editor system is associated with off-target effects on DNA and RNA.[161–163] These effects may be mitigated by further protein engineering of the base editing proteins used in these system.[161,162]

29 | COMBINING CRISPR EDITING WITH RNA-SEQ TO MEASURE COMPLEX PHENOTYPES

Molecular biologists can now use CRISPR-Cas9 systems to systematically test whether inactivation or activation of any single gene in the genome affects the viability of immortalized cell lines.[164–168] These screens use CRISPR systems to mutate (CRISPR) or use modified CRISPR systems to inhibit (CRISPRi) or promote (CRISPRa) transcription thereby modulating gene function. Next-generation studies are expanding the scope of what can be discovered through CRISPR screening on several fronts. Screens that modulate pairs of genes in each cell are being used to map functional genetic interactions in human cells, revealing buffering and synergistic relationships that produce synthetic lethality or disease suppression.[169] The number of cells required to screen all possible combinations of gene pairs exceeds present technical limitations, so interaction studies currently employ rationally selected sets of genes. CRISPR-Cas9 screens are also testing phenotypes beyond cell viability, obtaining much more detailed information about cellular response to perturbations through single-cell RNA sequencing methods, such as Perturb-seq.[170] Screening perturbations of gene pairs, with the phenotype read-out assessed by gene expression in individual cells via Perturb-seq, is revealing new aspects of how genes work together.[171]

30 | GENOME EDITING AT SCALE TO SIMULTANEOUSLY ASSESS ALL POSSIBLE MUTATIONS

Protein-altering variants can produce heritable prostate cancer risk by functional inactivation of a tumor suppressor, such as *BRCA2*. Some variants, such as introduced stop codons produce functional inactivation that is reliably predictable from sequence analysis alone. Other benign variants do not affect the protein sequence at all and can be reliably predicted not to affect cancer risk. However, many rare variants change the protein sequence in ways that are not clearly either benign or pathogenic. These variants of uncertain significance (VUS) present a dilemma for patients, clinicians, and investigators.[172] Computational approaches that predict the effects of VUS are informative, but these methods do not always agree and they provide insufficient evidence for clinical decision-making.[173]

Exhaustively testing the functional consequences of every possible amino acid change in a target gene is now becoming feasible. Multiplexed functional assays can determine the consequences of a large pool of VUSs by employing CRISPR-Cas9 editing to edit thousands of variants in a pool of cells. Each cell receives a single variant, and the pool of cells is subject to a functional assay such that the damaging variation can be detected through subsequent high-throughput sequencing of the cell pool. A recent study tested almost 4000 variants across *BRCA1* to predict which were deleterious.[174] This assay exploited the fact that the cell line HAP1, which harbors only a single copy of every gene, cannot tolerate inactivation of the cancer predisposition gene *BRCA1*. This method successfully predicted known pathogenic and benign alterations, made new predictions about which variants would be pathogenic, and generated sequence-function maps of how variants in sequence affect protein function. Multiplexed functional assays are being adopted to investigate driver genes in a variety of contexts.[175–178]

31 | FUNCTIONAL INTERROGATION OF GENOMIC REGIONS THAT AFFECT PROSTATE CANCER RISK

Functional screening is also revealing how regulatory regions outside of the coding genome affect prostate cancer risk. Commonly occurring inherited genetic variants are associated with small increases in prostate cancer predisposition.[179,180] Functional interrogation of these variants is complex, because the variant identified in association studies may be in genetic linkage with the causal variant, meaning it is physically nearby on the genome and co-inherited with the causal variant without being causal itself. Such variants have modest effects, and it is not obvious a priori in which cell type or when during tumor formation they are relevant. In contrast to mutations that inactivate the function of a protein-coding gene, such as *BRCA1*, common variants usually occur in noncoding regulatory regions and exert their effects at a distance.[180–182] Recent approaches to functionally interrogate these variants include CRISPR-Cas9-mediated inhibition of activity at regulatory regions to identify which loci influence gene expression.[183,184] Such expression quantitative trait loci (eQTL) can directly affect the expression of key driver genes, such as *MYC*. The effects of these risk loci are mediated by the epigenetic activity of the insulator protein CTCF, which binds to DNA regulatory regions. Integrative studies that combine CRISPRi, eQTL analysis, and assessment of CTCF activity are revealing the mechanisms by which germline variants affect prostate cancer risk.

32 | NON-CODING GENOMIC REGIONS AFFECT PROSTATE CANCER PROGRESSION AND DRUG RESPONSE

Quantitative assays that determine where and when transcription factors bind to regulatory DNA are revealing how these proteins affect prostate cancer progression. Many investigations have assessed how occupancy of DNA by transcription factors relevant to prostate cancer (AR, FOXA1, HOXB13) and histone marks associated with transcriptional control (H3K27ac, H3K4me2/3, H3K27me3) change across different stages of tumor development.[185,186] New studies are integrating genome and epigenome data from tissue samples obtained at all stages of human prostate cancer development. Unlike FOXA1 and HOXB13, the AR cistrome is altered between normal prostate, localized disease, and metastatic disease. This reprogramming in tumors largely reflects increased occupancy at sites occupied in normal tissue, rather than de novo binding at sites not observed in normal tissue. Mapping epigenetic reprogramming across human tumors and combining these data with structural variation data can identify key enhancers affecting *AR* and other genes.[187,188] Integrated analysis of how structural alterations, mutations, and epigenetic alterations alter protein function in tumors will reveal how these genetic and epigenetic forces shape tumor development and drug response.

33 | EXTRACELLULAR VESICLES: AGENTS AND INDICATORS OF TUMOR EVOLUTION

All cellular organisms release or shed extracellular vesicles (EVs).[189–191] EVs are not only a mechanism for discarding cellular debris but also a route of cellular communication via the

transfer of the cargo components borne by those vesicles.[192,193] Due to the ubiquity of EVs in biological systems, and due to the fact that the cargo of EVs reflects their cellular origin, EVs are an emerging area of research for studying tumor biology and for detection of tumors with liquid biopsies.

EVs as indicators of tumor evolution are a growing focus in all oncologic fields. Because most EVs are typically submicron in size, they are able to diffuse from the tumor microenvironment into peripheral biofluids, such as a serum, plasma, and urine. Therefore, EVs are of interest especially in liquid biopsies, with which EV proteins, RNA, and other cargo components are being investigated. The lipid bilayer structure of EVs protects internal cargo RNA and improves the stability of RNAs for testing in liquid biopsies.[194,195] Although circulating tumor DNA (ctDNA) and CTCs are associated most directly with certain stages of tumor cell evolution or death, EVs are commonly released as an active process at all stages of tumor progression, and tumor-specific genetic alterations may be detected in EVs. Although these are early days in our understanding of how to study and use EV-related data in the management of prostate cancer, significant progress is being made.

Not only are EVs a rich source of biologically informative data regarding tumor status, EV cargo may participate in the pathogenesis of tumor progression. PD-L1 expression on tumor-derived EVs is associated with immune evasion, for example.[196] In prostate cancer, large EVs, referred to as “large oncosomes,” ranging in size from 1 to 10 microns, appear to carry most of the tumor DNA that is circulating in prostate cancer patient plasma.[197,198] These large prostate cancer oncosomes also mediate changes in the tumor microenvironment and prostate fibroblast reprogramming, via AKT1-induced MYC activation.[199]

Because EVs in circulation are a heterogeneous mixture of EVs, new methods for identification and analysis of tumor-specific EVs, such as CSPG4-directed immunoaffinity-based isolation of tumor-derived EVs from patients’ plasma,[200] are being used to investigate relative immune suppression or immune activation by different tumor-associated EV populations. Methods for performing analyses of individual EVs remain challenging due to the heterogeneity of EV populations and limitations of tools available to characterize EVs accurately.[201–203] Selective capture and analysis of specific EV subsets, based on specific surface markers, such as CSPG4 (for certain tumor-derived EVs) or CD3 (for T cell-derived EVs), has proven to be a useful path forward in the field. In general, these studies altogether indicate that tumor-derived EVs may mediate disease and may be used to monitor disease. In the interplay between tumor and immune cells, tumor-derived EVs appear to drive multiple immune-inhibitory pathways[204]; $CD3^+$ EVs are associated with advanced tumor stage,[205] and EVs can be used to monitor responses to immunotherapy treatments.[206] Additional studies on the role of EVs in tumor progression and treatment responses, and their potential as predictive biomarkers, are warranted.

34 | CONCLUSION

The discussions at the 2019 CHPCA Meeting were the most active and abundant ever recorded over the history of this meeting (now in its 7th year), with 422 questions asked over 35 talks. We hope that the knowledge exchanged at this meeting and through the

dissemination of this meeting report will accelerate novel basic, translational, and clinical research that will deliver new and critical understandings into prostate cancer biology and how to improve treatment and outcomes for all men with prostate cancer.

The theme of the 2020 CHPCA Meeting will be: “Prostate Cancer Research in the 21st Century.”

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