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Prostate Cancer Research in the 21st Century; Report from the 2021 Coffey-Holden Prostate Cancer Academy Meeting

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Abstract

Introduction.—The 2021 Coffey - Holden Prostate Cancer Academy (CHPCA) Meeting, “Prostate Cancer Research in the 21st Century,” was held virtually, from June 24 – 25, 2021.

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SGZ has pending patent applications on molecular signatures in prostate and breast cancer licensed to Decipher Biosciences and Exact Sciences unrelated to this work.

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Methods.—The CHPCA Meeting is organized by the Prostate Cancer Foundation (PCF) as a unique discussion-oriented meeting focusing on critical topics in prostate cancer research envisioned to bridge the next major advances in prostate cancer biology and treatment. The 2021 CHPCA Meeting was virtually attended by 89 investigators and included 31 talks over nine sessions.

Results.—Major topic areas discussed at the meeting included: cancer genomics and sequencing, functional genomic approaches to studying mediators of plasticity, emerging signaling pathways in metastatic castration resistant prostate cancer (mCRPC), Wnt signaling biology and the challenges of targeted therapy, clonal hematopoiesis, neuroendocrine cell plasticity and anti-tumor immunity, cancer immunotherapy and its synergizers, and imaging the tumor microenvironment and metabolism.

Discussion.—This meeting report summarizes the research presented at the 2021 CHPCA Meeting. We hope that publication of this knowledge will accelerate new understandings and the development of new biomarkers and treatments for prostate cancer.

Keywords

therapeutics; precision medicine; cancer immunotherapy; tumor genomics; molecular imaging

Introduction

The Prostate Cancer Foundation (PCF) is a 501(c)(3) charitable organization that globally funds academic research focused on biology, biomarkers and treatments for aggressive and/or advanced prostate cancer. PCF also organizes an extensive global knowledge exchange program which includes annual scientific conferences including the CHPCA, and conducts other programs to develop new research partnerships and initiatives, and patient education measures.

The CHPCA Meeting is a discussion oriented invitation-only scientific conference that focuses on cutting edge research from prostate cancer and other intersecting fields that may accelerate new treatments for patients with advanced prostate cancer [1–7]. This Meeting, held ~annually, was named for prostate cancer research pioneers, Dr. Stuart Holden and the late Dr. Donald Coffey. The Meeting is organized by early career investigators, and attended by ~75–80 researchers, at least half of whom must be early career investigators. The Meeting format centers on discussion, with short talks followed by long discussion times, modeled after the former NCI Prouts Neck Meetings on Prostate Cancer [8].

The 2021 CHPCA Meeting, themed “Prostate Cancer Research in the 21st Century,” was held virtually from June 24 – 25, 2021. The Meeting was virtually attended by 89 investigators, including 53 young investigators (60%). There were 31 talks over 9 sessions, which focused on topics including cancer genomics, mechanisms of plasticity, new prostate cancer driver pathways that may function as treatment targets, clonal hematopoiesis, cancer immunology, optimizing prostate cancer immunotherapy, and novel molecular imaging agents to study the tumor microenvironment and cancer metabolism.

Stem-Like and Regenerative Properties of Luminal Cells

The normal prostate gland consists of basal and luminal epithelial cells and rare neuroendocrine cells that are surrounded by fibromuscular stroma and vessels [9]. With surgical castration, the prostate gland involutes to ~ 90% of its size, largely due to loss of luminal epithelial cells. However, upon the addition of exogenous androgens, within four weeks, the prostate gland can fully regenerate to its original size and biology [10]. To understand how the prostate changes during castration and regeneration, Karthaus *et al.* performed single cell RNA–sequencing of the murine prostate during a cycle of castration and regeneration. In an intact setting, the authors identified one basal and three luminal (L) epithelial cell subpopulations. The predominant (~96%) luminal subtype (L1), has a secretory phenotype and expresses high levels of canonical androgen receptor target genes. Rare L2 cells (~3%) are marked by *Sca1, Tacstd2,* and *Psca,* genes associated with progenitor cells [10]. Notably, L2 cells are predominantly located in the proximal duct, whereas L1 cells are in the distal ducts, suggestive of anatomically specific signaling niches determining luminal cell fates [10]. L3 cells (~1%) are defined by the transcription factor *Foxi1* [10], and resemble previously described ionocytes in the lung, a cell type that regulates salt balance, and has also been implicated in the pathophysiology of in cystic fibrosis [11]. Upon castration, profound transcriptomic changes were found in both the L1 and L2 subsets. Specifically, at day 28 of castration, L1 and L2 gained similar progenitor like transcriptomic features and increased proliferative indices in both L1 and L2 cell types during early regeneration, suggesting all luminal cells contribute to prostatic regeneration. This was elegantly confirmed by lineage tracing using a Rosa26/four–color Confetti allele with a luminal-specific *Krt8*–Cre^{ERT2} driver [10]. Additionally, similar observations were made in human luminal cells treated with androgen receptor signaling inhibitor (ARSI). While this study focused on the anterior–posterior (AP) lobe, other groups have reported similar cellular compositions in the other lobes of the murine prostate [12, 13] *albeit* with distinct biology of L1 cells of each lobe. In addition to these lobe specific differences, Guo *et al.* further showed the oncogenic potential of L2 cells. Using a L2 specific *Krt4*$^{CreERT2/+}$; *Rosa26*$^{tdTomato/+}$; *Pten*$^{fl/fl}$ mouse, PIN lesions were noted at 2 months post tamoxifen administration [12]. Overall, these studies highlight the importance of luminal cell complexity in prostate regeneration and hint at how different cells of origin can be more or less susceptible to tumor formation. Future studies should examine the similarity of these luminal subtypes to their human counterparts in more granularity, and their impact on prostate cancer progression and plasticity.

Prostate Cancer Genomics and Sequencing

Genomics provides a tool which can be used to shed light into the molecular underpinnings of prostate cancer. The decreasing price and increasing clinical and research adoption of next generation sequencing (NGS) in prostate and other cancers has created enormous amounts of NGS data which grows daily. This increase in data is not isolated to NGS, as electronic medical records (EMRs) have also multiplied the amount of unstructured clinical data per patient. This provides an opportunity to apply machine-learning approaches, and specifically, interpretable deep learning approaches to try and gather important insights from this flood of data. Making sense of this diverse data requires integration of clinical NGS data, computational approaches, and our biological understanding of oncologic processes.

Historically, approaches have focused on individual genes [14–16]. Newer studies and especially single-cell sequencing have enabled the step from genes to pathways/programs and how they interact with each other [17, 18], for example the divergent programs in prostate adenocarcinomas vs. neuroendocrine prostate cancer (NEPC). Furthermore, the spatial organization within a tumor needs to be taken into account [19]. All of this NGS data ideally will be linked to the clinical history of an individual patient in order to provide the complete context for the genomic findings and provide actionable results at the point of care. Many major academic medical centers have active clinical and research NGS programs. However, the patient population at these institutions is highly self-selected, and it is important to expand beyond these institutions into the community, where most patients are ultimately diagnosed and treated. The team at Metastatic Prostate Cancer Project (mpcproject.org) have developed a nationwide genomic research study which will generate a comprehensive NGS database. Patients will be able to donate archival tissue, saliva, blood, and allow access to medical records. The goal of this effort is to bring research to patients outside of NCI-designated cancer centers, thereby accelerating discoveries. As of June 2021, over 1,000 men have enrolled in the project, and the team is actively working on ongoing data releases for the entire community via cBioPortal and other forms of data sharing.

With the advent of CRISPR functional genomics technology, genomics has moved beyond being just a descriptive tool. In particular, CRISPR screens allow for high-throughput assessment of gene regulation [20–23]. A CRISPR screen of the androgen receptor (AR) paired with a newly developed endogenous AR reporter has revealed known and novel therapeutic targets that represent potential druggable alternatives to AR itself (Li, Gilbert, Feng, et al., unpublished). In addition, prostate cancer is generally thought to be immunologically cold, which is one of the hypothesized reasons why immune checkpoint blockade has had lukewarm efficacy in unselected populations. MHC-I silencing is another potential contributor to prostate cancer immune evasion. Using a similar approach, a CRISPR screen using a fluorescent antibody-based approach has identified additional targets which may increase MHC-I expression and suggest potential therapeutic strategies to increase the efficacy of immunotherapy in prostate cancer (Chesner, Gilbert, Drake, Feng, et al., unpublished). These approaches demonstrate the power of functional genomics to accelerate our ability to make translational discoveries at scale.

Clonal Hematopoiesis

Clonal Hematopoiesis in Cancer Patients—Clonal hematopoiesis (CH) denotes the presence of somatic, leukemia-associated mutations in hematopoietic cells, in absence of overt hematologic malignancy [24]. CH is a common occurrence with aging, and predicts an increased risk of subsequent hematologic cancers and increased mortality, particularly from cardiovascular disease [25–27]. CH is particularly important among individuals with solid tumors given that it leads to an increased risk for therapy-related myeloid neoplasms upon exposure to cytotoxic therapies [28–31]. In a large study of 24,146 advanced cancer patients who underwent paired tumor and blood next-generation sequencing, 30% of patients had CH [32]. Among individuals who had prior therapy for their cancer, CH was more commonly identified, and these individuals had an enrichment for somatic alterations in genes in the DNA damage response pathway including *PPM1D*, *TP53*, and *CHEK2*

[32]. CH was associated with prior radionuclide therapy, external beam radiotherapy, and cytotoxic chemotherapy, with higher cumulative exposures increasing the presence of CH [32]. Among newer targeted therapies, and relevant to prostate cancer, PARP inhibitors lead to an increased risk for therapy-related myeloid neoplasms [33, 34]. Individuals having received prior PARP inhibitors demonstrated an increased risk for CH, though this association weakened when adjusting for additional prior therapies such as platinum agents [35]. Ongoing work with prospective serial sequencing may help to better elucidate this association, especially for prostate cancer patients who are more often naïve to cytotoxic chemotherapy. As CH is currently not an intervenable state, routine testing is not advised, though ongoing clinical trials hopefully may change the landscape of CH as an unmodifiable entity to one that can lead to early interventions to benefit patients from its adverse consequences.

Clonal Hematopoiesis' Impact on Plasma Cell Free DNA (cfDNA) Testing— CH can complicate interpretation of cfDNA testing as well. Studies of normal individuals have demonstrated CH serves as a source of "biological background" in cfDNA analyses, being present in 63% of individuals tested [36], implying that cfDNA may not be specific for cancer-associated alterations. Subsequent work of paired white blood cell (WBC) and cfDNA testing, including both individuals with and without cancer (cancer types including prostate, breast, and lung cancer) utilizing a more sensitive assay covering a larger genomic footprint, demonstrated the majority of mutations detected from cfDNA testing originate from CH (81.6% in controls and 53.2% in cancer patients) [37]. CH was also evident in >90% of WBCs from both cancer patients and healthy controls. Age of patient was highly correlated with the presence of CH and number of mutations detected [36, 37], though age was not a predictor for CH in another cfDNA study of prostate cancer patients, possibly influenced by the older cohort leading to a lack of discrimination [38]. *PPM1D* mutations were particularly enriched among patients who had prior exposure to chemotherapy and/or radiation therapy [37]. A prostate-cancer specific study examined this phenomenon and found CH variants in 19% (13 of 69) of men tested, which has clinical relevance when selecting candidates for PARP-inhibitor therapy, given that 10% (7 of 69) of individuals had CH mutations in relevant genes (*ATM*, *BRCA2*, and *CHEK2*) [39]. Though there are strategies to adjudicate probable CH [39, 40], in absence of paired WBC sequencing, CH poses a major pitfall regarding interpretation of cfDNA analyses for individuals with all malignancies.

Epigenetic and Transcriptional Targets of Lineage Plasticity

The concept of sustained oncogenic addiction to androgen receptor (AR) after hormonal therapy has led to numerous therapeutic advances in prostate cancer [41, 42]. While these next–generation AR therapies have greatly improved patient outcomes, a growing subset of prostate cancers no longer depend on AR for survival and represent a major unmet clinical need [43]. This subset of resistant prostate cancers is enriched for the loss of *TP53* and *RB1* [44, 45] and can also often transform from adenocarcinoma into NEPC—the latter concept is known as lineage plasticity [44]. Many investigators have highlighted the activation of stem–like and epigenetic transcription factors in lineage plasticity, including the upregulation of *SOX2*, *SOX11*, *EZH2, LSD1,* and *BRD4* [46–50], and a number of

such targets have been at the forefront of therapeutic development. The study which was presented at this forum further explored factors that may contribute to lineage plasticity in prostate cancer and implicated (BET) bromodomain proteins and the transcription factor E2F1 [51]. *Kim et al.* utilized transformed NEPC LnCAP cell lines (MR42D and MR42F) to demonstrate through integrative RNA and ChiP based experiments that E2F1 cooperates with BRD4 to induce an AR–repressed lineage plasticity program. BET inhibition blocked this program and decreased the growth of several *in vitro* NEPC models [51]. The authors also validated the clinical relevance of *E2F1* and *BRD4* upregulation in a recently published phase 1 clinical trial of ZEN-3694, a BET inhibitor, which was tested in combination with enzalutamide in men with metastatic CRPC who previously progressed on enzalutamide or abiraterone [52]. Out of 13 baseline biopsies with tumor tissue present, four patients were found to have transformed to NEPC. Two out of the four subjects with high levels of *E2F1* and *BRD4* mRNA expression also had prolonged disease control with ZEN-3694 (168 weeks and 40 weeks) [51]. This study, among others [53], highlights the potential efficacy of targeting epigenetic and transcriptional regulators in a small subset of late–stage tumors that have undergone lineage plasticity; however, it also raises the need to dissect the early events that may predispose specific adenocarcinoma tumors to undergo a transition to neuroendocrine prostate cancer to address the high degree of late–stage inter–tumoral heterogeneity.

The Relationship of MYC and AR

Overexpression of *c*–MYC (MYC) is observed in luminal cells of prostate intraepithelial neoplasia (PIN) lesions and a large proportion of adenocarcinomas [54]. Furthermore, *MYC* amplifications are enriched in metastatic CRPC compared with castrate–sensitive prostate cancer (CSPC) [45]. Human *MYC* has been shown to be sufficient to initiate prostate tumors. Transgenic mice overexpressing human *MYC* under the prostate epithelium-specific ARR2Pb promoter develop PIN that transition to cancer at 3 to 6 months, and invasive cancer after 6 months with aberrant tumor vasculature. Castration has been found to have a profound effect on tumor regression, with resumed growth upon androgen replenishment [55]. To understand the ongoing yet elusive interplay between MYC and AR, Qui and Boufaied *et al.* first performed *s*ingle cell RNA sequencing on the ARR_2Pb–MYC transgenic mice at 12 weeks of age. Major transcriptomic changes were noted in *MYC* transformed luminal cells with a negative impact on the AR–dependent transcriptional program. AR ChIP–Seq showed a distinct AR cistrome in MYC transformed cells with an expansion of >1,500 sites [54]. To delineate the AR and MYC interplay, an RNA Pol II ChIP-seq approach was used that determined transcription factor rates as a function of RNA Pol II occupancy across the genome. In MYC overexpressing lesions, AR transcriptional targets demonstrated higher RNA Pol II transcriptional pausing [54]. These findings serve as a potential mechanism for MYC mediated transcriptional repression at AR–regulated genes. Furthermore, as concurrent low AR and high MYC transcriptional programs predicted for a shorter time to biochemical recurrence and progression to metastatic CRPC, this study was suggestive of *MYC* conferring resistance to ARSIs. In addition to *MYC,* the study highlights the need to further understand interactions between AR and amplified oncogenes, such *FOXA1* and *ERG* [56, 57]. Such interactions will better delineate how AR cooperativity with commonly perturbed or amplified transcription factors drives prostate cancer resistance

to ARSIs. Furthermore, while this study focused largely on tumor intrinsic mechanisms of AR and MYC regulation, unraveling changes in macrophage infiltration with and without castration in the Hi-MYC model [58], and studying the effect of macrophage depletion on tumor regression will be necessary to define tumor-microenvironment-mediated protective and pro-oncogenic signals.

Emerging Signaling Pathways in mCRPC

Targeting Autophagy Potentiates Immune Checkpoint Blockade in Prostate Cancer Therapy—Autophagy is a cellular degradation pathway for the clearance of damaged proteins and organelles under stressful conditions. It provides an alternative energy source during nutrient deprivation to maintain homeostasis and viability [59]. Several studies suggest that autophagy protects tumors from necrosis and inflammation in response to metabolic stress [60, 61], and autophagy inhibition may sensitize tumors to immune checkpoint inhibitors through immunomodulatory mechanisms [62, 63]. Most recently, a study reported that ESK981, a phase I–cleared multi-tyrosine kinase inhibitor with a novel autophagy inhibitory property, suppressed tumor growth in CRPC and potentiates responses to immune checkpoint blockade by anti-PD-1 [64]. Mechanistically, ESK981 directly targets the lipid kinase PIKfyve to inhibit autophagy, meanwhile it upregulated expression of the chemokine CXCL10 through the interferon-γ pathway and promoted functional T cell infiltration. Genetic inhibition of PIKfyve recapitulated ESK981's antitumor activity and enhanced the efficacy of anti-PD-1 therapy through activation of interferon responses [64]. This study reveals that inhibition of autophagy by targeting PIKfyve via ESK981 may prime the tumor immune microenvironment and be an effective therapeutic strategy alone or in combination with immune checkpoint blockade in patients with advanced prostate cancer.

Ferroptosis and Prostate Cancer—Ferroptosis is a unique iron-dependent form of non-apoptotic cell death and is morphologically, biochemically, and genetically distinct from apoptosis, necrosis, and autophagy [65]. Like glutamate, the oncogenic RAS-selective lethal small molecule erastin inhibits cystine uptake by the cystine/glutamate antiporter (system x(c)(−)), creating a void in the antioxidant defenses of the cell and ultimately leading to oxidative cell death. Ferroptosis sensitivity is regulated by the composition of membrane phospholipids, especially the amount of polyunsaturated fatty acids (PUFAs) versus monounsaturated fatty acids (MUFAs) found in these lipid species. Less oxidizable MUFAs compete with more oxidizable PUFAs for insertion into membrane phospholipids, thereby governing the sensitivity of the membrane to oxidative destruction [66]. DECR1, an enzyme involved in the catabolism of PUFAs, is robustly overexpressed in prostate cancers and is associated with worse survival [67]. One model is that DECR1, which is negatively regulated by AR, limits the amount of PUFA oxidation and thereby prevents tumor cells from undergoing ferroptosis [67]. Indeed, targeting DECR1 causes cellular accumulation of PUFAs, enhanced mitochondrial oxidative stress and lipid peroxidation, and induced ferroptosis [67]. In another study, enzalutamide was found to induce extensive phospholipid remodeling and increase membrane PUFA levels, causing hypersensitivity to ferroptosis [68]. These studies provide insight into development of novel therapeutics for advanced prostate cancer that target lipid metabolism and the ferroptosis pathway.

Wnt Signaling and the Challenges of Targeted Therapy

Wnt Signaling Drives Distinct Pathways in Primary and Metastatic Prostate Cancer—Wnt signaling in prostate cancer has experienced recent renewed interest with the findings by the PCF-SU2C International and West Coast Prostate Cancer Dream Teams that the Wnt/β-catenin pathway is commonly altered and is the top differentially-regulated pathway among enzalutamide resistant CRPC patients [69, 70]. It is known that genomic alterations of a number of genes can activate canonical Wnt/β-catenin signaling (ie APC, β-catenin, RSPO, RNF43, ZNRF3) and drive tumor progression. However, unlike other cancers such as colorectal carcinoma and certain subtypes of hepatocellular carcinoma and leukemia where Wnt family genomic alterations predominate, only ~20% of advanced CRPC tumors have alterations in these genes [70] and their contribution to prostate cancer progression is uncertain.

An important step in addressing this uncertainty is defining a transcriptomic signature for canonical Wnt/β-catenin signaling in prostate cancer. Recent work combining the transcriptomes of prostate cancer cell line models with knockdown of the Wnt signaling inhibitory proteins APC, and RNF43 and stimulation with Wnt3a, has elucidated a 47 gene signature for canonical Wnt/β-catenin signaling (Balk et al., unpublished). This signature contains many well-known canonical Wnt/β-catenin signaling target genes (AXIN2, LEF1, CD44, and ZNRF3), but also additional novel genes (AHR, RUNX1, ROR1) (Balk et al., unpublished). Based on the 47 gene signature, a canonical Wnt/β-catenin signaling activity score was applied to the TCGA dataset of primary prostate cancer. Tumors with APC loss had the highest Wnt scoring tumors, but interestingly a large number of primary tumors exhibited a high canonical Wnt/β-catenin activity score without having obvious Wnt-activating genomic alterations (Balk et al., unpublished). This suggests that Wnt/β-catenin signaling activity in many primary tumors may be driven by epigenetic mechanisms. In comparison, advanced CRPC tumors in the PCF-SU2C dataset generally had a much lower canonical Wnt/β-catenin activity score, with the highest activity also being found in tumors with APC loss (Balk et al., unpublished). In primary prostate cancer, one of the transcripts that correlates most strongly with high canonical Wnt/β-catenin signaling is the carrier protein WLS (Wntless, GPR177) (Balk et al., unpublished). WLS binds and assists in vesicular transport of palmitoleated Wnt ligands to the cell surface for secretion and recently has been implicated in driving prostate cancer resistance to enzalutamide [71]. Interestingly, despite the comparatively lower level of canonical Wnt/β-catenin signaling found in advanced CRPC tumors in the PCF-SU2C data set, WLS protein is expressed at intermediate to high levels in ~70% of these advanced CRPC tumors, suggesting high levels of Wnt ligand secretion (Balk et al., unpublished). One possible explanation is that the high level of WLS expression in advanced CRPC tumors is indicative of Wnt ligand secretion driving non-canonical rather than canonical Wnt signaling. This is supported by a recent study that implicates WLS in the activation of non-canonical ROR2/PKCδ/ERK signaling to promote the castration-resistant NEPC phenotype [72]. Significantly, this study as well as other work presented at the meeting found that prostate tumors with high WLS expression are highly sensitive to inhibitors of porcupine (PORCN) (ie LGK974, ETC159) (Balk et al., unpublished), an O'acyltransferase which is required for Wnt ligand secretion.

Pre-Clinical Studies for Advancing Cirmtuzumab-Based Anti-ROR1 Therapies in Metastatic Prostate Cancer—Non-canonical Wnt signaling that does not rely on β-catenin for transducing a downstream signal is thought to play a role in the advanced, metastatic CRPC setting [73]. WNT5A is one of the main Wnt ligands considered to mediate non-canonical Wnt signaling. Studies in prostate cancer have shown WNT5A is a critical factor in prostate development, can stimulate the invasive properties of prostate cancer cells in culture, and is a marker of poor prognosis when increased in circulating tumor cells of patients with bone metastatic CRPC [74–77]. WNT5A binds to the non-canonical Wnt signaling receptor, ROR1, which is not normally expressed outside of development, but can become re-expressed in multiple cancer types. WNT5A stimulation of ROR1 has been shown to stimulate activation of Rho-GTPases and Rac1/2 leading to leukemia cell proliferation, an effect that can be blocked by the anti-ROR1 humanized monoclonal antibody, Cirmtuzumab [78]. Preclinical studies presented at the meeting showed increased expression of ROR1 in several cell lines (PC3, DU-145) and a patient-derived xenograft (PDX) model (PCSD13) of NEPC. Pretreatment of the PCSD13 PDX with Cirmtuzumab increased the efficacy of docetaxel at inhibiting tumor growth. These studies have formed the rationale for development of a phase 1b clinical trial of Cirmtuzumab in combination with docetaxel in metastatic CRPC patients. Efforts are also underway to develop an anti-ROR1 chimeric antigen receptor T cell (CAR-T) therapy. Pilot studies of intravenous infusion of anti-ROR1 CAR-T cells into mice bearing subcutaneous ROR1-expressing PC3 xenografts, showed marked reduction in tumor growth.

Targeting Hyperactive Wnt in Colon and Prostate Cancer—Analysis of sequencing data from primary prostate cancer (TCGA dataset) and advanced CRPC (PCF-SU2C dataset) show that prostate cancer tumors harbor genomic alterations in canonical Wnt signaling family members, including truncating mutations in APC, hotspot mutations in β-catenin, and increases in LRP5 and LRP6 amplifications [79]. However, closer examination suggests that increased genomic alterations in canonical Wnt signaling family members are a feature of metastases, rather than castration-resistance, as both metastatic hormone-sensitive and castration-resistant prostate cancer show these alterations [45, 79]. In preclinical mouse models, APC knockout in $PTEN^{-/-}$ or $p53^{-/-}$ mouse prostate cancer organoids enhanced tumor growth following orthotopic transplantation, and greatly increased their ability to metastasize following tail vein injection [79]. These results suggest hyperactive canonical Wnt/β-catenin signaling can drive both prostate cancer proliferation and invasiveness. APC truncating mutations are more common in advanced CRPC than APC losses. Significantly, a comparison of colorectal and prostate cancer reveals that in prostate cancer, APC truncating mutations tend to be shifted C-terminally compared with the mutation cluster region in colorectal cancer. This shift results in prostate cancer expressing truncated forms of APC that maintain a 20 amino acid (aa) repeat motif found in the mutation cluster region. Previous work in colorectal cancer has shown that APC truncating mutations that maintain this 20aa repeat motif are better able to bind the GSK3/APC/Axin destruction complex that degrades β-catenin and have much better responses to tankyrase inhibition [80]. In theory this suggests that prostate cancers driven by APC truncating mutations are more likely to respond to tankyrase inhibitor therapies, providing a novel therapeutic approach. However, it is important to note that while targeting hyperactivated

Wnt signaling in most preclinical colorectal cancer models was effective, resistance to Wnt-targeted therapies could develop from accumulation of additional genetic alterations (KRAS, BRAF activations, p53, SMAD4 disruptions, YAP/TAZ activation) that can lead to lineage reversion and Wnt independence [81]. Whether similar resistance mechanisms would develop in primary prostate cancer or CRPC remains to be determined.

Precision Therapeutics for Lethal mCRPC: The Case for DKK-1—The recent success of immune-checkpoint blockade therapies in melanoma as well as renal and lung carcinomas has sparked interest within the prostate cancer field to combine immune modulatory therapies with standard CRPC treatments. This has now been extended to include targeting of the Wnt signaling cascade to modulate the immune microenvironment. DKK-1 (Dickkopf-1) is a secreted protein that blocks Wnt signaling by binding to and isolating the Wnt LRP6 co-receptor that is required for FZD receptor transduction of Wnt ligand-induced signaling. DKK-1 is highly upregulated in advanced, AR-negative CRPC. As a secreted protein, DKK-1 can act on cells in the surrounding tumor microenvironment, including immune cell populations. Studies have shown an association of high DKK-1 levels with increased levels of myeloid-derived suppressor cells (MDSCs), as well as low levels of $CD8^+$ T cells in tumors [82]. Using the PCF-SU2C advanced CRPC dataset, a recent report demonstrated that CRPC tumors with high expression of DKK-1 also exhibit transcriptomic profiles consistent with immune evasion including increased M2 macrophages, decreased $C8^+$ T cells, and a shift toward increased levels of quiescent compared to activated natural killer (NK) cells [83]. Further, treatment of DKK-1 expressing PC3 xenografts with a DKK-1 neutralizing monoclonal antibody in NK cell proficient SCID mice led to a reduction of tumor growth, while treatment of the same type of xenograft in NSG mice that lack NK cells had no effect. The results of this study have formed the rationale for a phase 1b/2a clinical trial of a DKK-1 neutralizing monoclonal antibody (DKN-01) as monotherapy or in combination with docetaxel in patients with DKK-1 positive, advanced CRPC (NCT03837353). Syngeneic mouse models are being used to further interrogate the impact of DKK1 and canonical Wnt signaling on the tumor microenvironment.

Strategies to Mitigate Bone Toxicity when Targeting Wnt/B-Catenin Signaling —Wnt signaling is broadly active across many different cell types and tissues so it is no surprise that therapies targeting Wnt signaling in cancer can have unintended toxicities in tissues that are especially reliant on Wnt signaling (ie bone and intestinal mucosa). In particular, bone toxicity in the form of progressive bone loss induced by Wnt inhibition has been a major issue requiring attempts at mitigation. In preclinical mouse models, simultaneous treatment with bisphosphonates such as alendronate have been successful in mitigating bone resorption caused by the PORCN inhibitors, LGK974 and ETC-159 [84]. These results have provided a conceptual strategy to reinitiate PORCN inhibitor trials previously put on hold to due to progressive bone loss with the addition of bisphosphonates or anti-RANKL monoclonal antibodies to the treatment regimen.

An alternative strategy for mitigating the toxicities involved with broad Wnt inhibition is developing therapies that narrowly target certain types of Wnt signaling. PORCN inhibitors shut down secretion of all 19 Wnt ligands and thus can impact the interaction of many

different types of Wnt ligand:FZD receptor interactions in both canonical and non-canonical Wnt signaling. Similarly, monoclonal antibodies that target multiple FZD receptors such as Vantictumab (anti-FZDs 1, 2, 5, 7, and 8) can have the same effect. Narrowing the therapeutic target can go a long way towards mitigating side effects as seen in the work described above targeting ROR1 and DKK-1 to block specific types of Wnt signaling [78, 82]. Efforts are currently underway to link expression of specific FZD receptors and R-spondins to both better and worse outcomes in clinical trials of broad Wnt inhibitors and thereby separate those FZD receptors with on-target treatment effects from those with tissue specific side-effects and toxicities.

Cancer Immunotherapy and its Synergizers

Targeting MYC to Enhance Immunotherapy—The importance of c-MYC as an oncogenic driver of cancer onset and tumor progression has been well studied in several solid tumor types [85, 86]. In mCRPC, MYC over-expression has especially been documented but the challenge has been on how to effectively target MYC. More recently, MYC has also been identified as a regulator of the anti-tumor immune response [87]. Due to the paucity of direct MYC inhibitors with *in vivo* activity, Abdulkadir and colleagues developed novel MYC inhibitor analogs that target the MYC/MAX complex [88, 89]. These MYC inhibitors bind to the b-HLH region to disrupt MYC/MAX complex formation and GSK3-beta-mediated phosphorylation of MYC resulting in MYC degradation [88]. MYC inhibition leads to an increase in immunogenic cell death followed by release of danger-associated molecular patterns (DAMPs), turning immunologically ‘cold’ prostate tumors ‘hot’. In MYC-expressing prostate cancer murine models (MycCaP), MYC inhibitor treatment increased tumor infiltration by NK and T cells as well as upregulation of PD-L1 expression on tumors in vivo [88]. Consequently, these tumors were sensitized to anti-PD-1 immunotherapy [88]. Further development of these novel MYC inhibitors is ongoing.

Elucidating Mechanisms by which Racial Differences Impact Drug Responsiveness and Anti-Tumor Immunity—Prostate Cancer remains a health disparity among African-American men and men of direct African descent [90]. Non-hormonal treatment options for castration-resistant disease have a very modest palliative and survival benefit, so the development of other treatment options is essential. There is great interest in targeting metabolic pathways that may be altered during prostate cancer progression. Specifically, activation of lipid metabolism has been described for most localized and metastatic prostate tumors, emphasizing its potential role in tumorigenesis and tumor progression. The 5’-AMP activated protein kinase (AMPK) has been described as a master regulator of lipogenic pathways and intracellular oncogenic signaling, however, the effects of metabolism on immune responses to prostate cancer have yet to be clearly delineated. Natural killer T (NKT) cells recognize lipid antigen presented by CD1d molecules, and prostate cancer cell lines express CD1d. Webb and colleagues have previously demonstrated that activation of AMPK results in increased CD1d-mediated NKT cell activation [91]. Studies from other groups have shown that drugs that activate AMPK such as metformin and aspirin have anti-tumor activity [92]. Therefore, Webb *et al.* hypothesized that modulation of NKT cells with metformin may sensitize prostate cancer cells to NKT mediated anti-tumor immunity. To test this hypothesis, prostate cancer cell

lines derived from European-American men (DU-145 and PC3) and African-American men (MDA-2a and MDA-2b) were treated with a panel of AMPK activators. Pretreatment of European-American prostate cancer cell lines with AMPK activators, such as metformin, resulted in a 2-fold increase in NKT cell responses, whereas responses to African-American cell lines remained unchanged (Webb *et al.,* unpublished). Whether single nucleotide polymorphisms (SNPs) in the AMPK-mTOR pathway may contribute to racial differences observed in treatment responsiveness was investigated. Twenty-two SNPs in the AMPK-mTOR pathway with higher allele frequencies in African-American men were identified, but the majority of the SNPs had no associated clinical significance. Future studies will employ fast photochemical oxidation of proteins (FPOP) to identify differences in proteomic profiles following drug treatment. Altogether, these data suggest a role for personalized therapy, given that ethnicity related differences can impact drug responsiveness and immune responses within the tumor microenvironment.

Unexpected Targets and Biomarkers of Checkpoint Immunotherapy—The success of immunotherapy in some cancer types, most notably inhibitors of the negative regulatory T cell checkpoints PD-1 and CTLA4, have led immunotherapy to be deemed the fourth pillar of cancer treatment [93, 94]. However, anti-PD-1/PD-L1 and anti-CTLA may have mechanisms of action that extend beyond their impact on effector T cells. To investigate the role of PD-1 outside of the T cell compartment, Boussiotis *et al.* generated mice lacking PD-1 in T cells (PD-$1^{f/fCD4Cre}$) and implanted them subcutaneously with various murine tumor cell lines [95]. In PD-$1^{f/fCD4Cre}$ mice, PD-1 expression was observed on myeloid progenitor cells that have the properties of monocytic and granulocytic MDSCs [95]. These cell types are produced from the bone marrow during emergency myelopoiesis which can be stimulated by tumor-derived growth factors. The role of PD-1 and its ligand, PD-L1, in myeloid progenitor cells and in tumor-driven emergency myelopoiesis was investigated. In wild type (WT) mice, there was very low expression of PD-1 and a modest level of PD-L1 on common myeloid progenitor (CMP) cells and granulocyte/macrophage progenitor (GMP) cells [95]. Following tumor implantation in WT mice, PD-1 expression was up-regulated in CMP and GMP cells, and these cell compartments were expanded, demonstrating tumor-driven emergency myelopoiesis [95]. In tumor-bearing PD-$1^{-/-}$ mice, cellular expansion of CMP was retained, but the GMP compartment was not expanded compared with WT tumor-bearing mice [95]. Lack of GMP accumulation was not found to be due to a blockade of cellular differentiation from CMP to GMP, rather there was an increase in output of effector myeloid cells in tumor-bearing PD-$1^{-/-}$ mice. Moreover, myeloid cells from tumor-bearing PD-$1^{-/-}$ mice lacked immune-suppressive function as evidenced by lack of nitric oxide (NO) secretion and inability to suppress proliferation of ovalbumin-specific T cells [95]. To study the role of PD1 specifically in myeloid cells, mice with PD-1 deletion in the myeloid compartment were generated (PD-$1^{f/fLysMcre}$). PD-$1^{f/fLysMcre}$ mice bearing tumors exhibited a decrease of GMP in the bone marrow [95]. Interestingly, while mice lacking PD1 in T cells exhibited some reduction in tumor growth, the growth of tumors was completely suppressed in mice lacking PD-1 in myeloid cells [95]. Mice lacking PD-1 specifically in $FoxP3^{+}$ regulatory T cells (Tregs) were generated to evaluate the role of PD-1 in this cellular subset (Pdcd$1^{f/fFOXP3}$ mice). PD-$1^{-/-}$ Tregs exhibited a more activated phenotype and a more potent suppressor capacity compared

to WT Tregs [95]. Together, these data demonstrate that PD1-expressing myeloid cells are associated with tumor-driven emergency myelopoiesis and increased tumor growth. Blockage of the PD1 axis in myeloid cells and Tregs may be important contributors to the efficacy of anti-PD-1/PD-L1 checkpoint immunotherapy in cancer patients. Whether PD-1 expression in these suppressive immune cell subsets may serve as a biomarker for responsiveness to checkpoint immunotherapy is an important question.

Chromosomal Instability and cGAS-STING: Friend or Foe?—The recognition of microbial nucleic acids serves as a major mechanism by which the innate immune system detects DNA-containing pathogens. cGAS is a cytosolic DNA sensor that activates innate immune responses through synthesizing second messenger cyclic GMP-AMP (cGAMP), which activates the stimulator of interferon genes (STING) [96]. In tumors, the cGAS-STING innate immune pathway can be triggered by tumor-derived DNA and generates antitumor immunity. Chromosomal instability (CIN) is a hallmark of cancer and is associated with tumor evolution, poor prognosis, and metastasis [97, 98]. CIN results in inappropriate double-stranded DNA (dsDNA) accumulation in the cytosol, resulting in constitutive activation of the cGAS–STING pathway [97]. Cytosolic dsDNA sensed by cGAS causes the production of cGAMP, activates STING and downstream noncanonical NF-κB signaling, which in turn promotes metastasis [99]. However, how chromosomally unstable tumor cells cooperate with chronic activation of innate immune pathways to evade immune surveillance remains unknown [97]. A recent study showed that the ectonucleotidase ENPP1 plays a pro-metastatic role by degrading extracellular cGAMP and producing immune suppressive adenosine [100]. In human cancers, ENPP1 overexpression promotes migration and metastasis of chromosomally unstable tumors, suppresses immune cell infiltration, and renders sensitive tumors resistant to immunotherapy [100]. Thus, ENPP1-induced cGAMP hydrolysis facilitates chromosomally unstable tumors to transmute cGAS activation into an immune-suppressive pathway.

Neuroendocrine Cell Plasticity and Anti-Tumor Immunity: Lessons from Lung Cancer

Responses to checkpoint immunotherapy are often highly correlated with tumor mutation burden, with relatively high objective response rates seen for melanoma, MMR-deficient tumors (colorectal and non-colorectal), and cutaneous squamous cell cancer, and low response rates seen in prostate cancer [101]. In non-small cell lung cancer (NSCLC), objective response rates to anti-PD-1/PD-L1 monotherapy range from 15–25%, often with durable activity. However, durable responses to checkpoint immunotherapy in certain lung cancer subtypes including small cell lung cancer (SCLC) and *KRAS-LKB1* (KL) mutant NSCLC are rare, despite a high mutational burden [101, 102].

SCLC is a highly plastic neuroendocrine (NE) cancer subtype that can arise either *de novo* or via trans-differentiation from adenocarcinoma. SCLC and *KRAS-LKB1* mutant NSCLC were found to evade anti-tumor immune responses by silencing activation of the cGAS-STING pathway, which activates interferon (IFN) responses upon detection of cytoplasmic double-stranded DNA [103, 104]. While NE SCLC cell lines typically grow in suspension *in vitro*, a subset of SCLC cells were found to have an adherent mesenchymal phenotype and expressed high levels of PD-L1; this phenotype could also be induced via

treatment of parental SCLC cells with HGF, and they could revert spontaneously, suggesting an epigenetic regulatory mechanism [105]. Parental PD-L1-low NE SCLC cells were found to repress STING and IFN signaling via epigenetic silencing of a subclass of endogenous retroviral coding sequences, while the PD-L1-high mesenchymal SCLC subset exhibited de-repression of endogenous retroviral genes and consequent activation of STING expression and IFN pathway activity [105].

NE SCLC cell lines commonly express low levels of MHC-I [106–108]. However, mesenchymal subsets expressed high MHC-I and presented a broad range of immunogenic peptides [108]. Consistent with these *in vitro* studies, evaluation of a panel of primary SCLC samples by IHC found that while most tumor cells lacked MHC-I, pockets of MHC-I-high tumor cells could be found which exhibited an altered morphology and had downregulated expression of NE genes such as ASCL1 and chromogranin. Thus, downregulation of NE markers is associated with recovery of MHC-I expression. Although durable responses to checkpoint immunotherapy in SCLC are rare, study of such cases may provide insights into their unique biology and biomarkers of response. In one case, a patient with *RB1*-loss, *TP53*-mutated MHC-I-high SCLC experienced a durable and deep response lasting over 6 years following 3 cycles of nivolumab + ipilimumab (which had been stopped due to panniculitis). In a Dana-Farber Cancer Institute cohort, overall survival was significantly higher in MHC-I-high (N = 7) vs MHC-I-low (N = 24) SCLC patients who had been treated with checkpoint immunotherapy. These data suggest that MHC-I expression levels may be a biomarker of response to checkpoint immunotherapy in SCLC.

Fundamental regulators of antigen presentation and SCLC epigenetic plasticity were next evaluated in mesenchymal (MHC-I-high) SCLC cells by identifying loci with gain of H3K27 acetylation coupled with loss of H3K27 methylation, and with upregulated expression on RNA-Seq, compared with parental (MHC-I-low) SCLC cell lines. Top upregulated genes in MHC-I-high vs MHC-I-low SCLC that were validated in human tumor samples included TAP1, which functions to deliver cytosolic peptides to MHC-I in the ER, as well as AXL. A panel of SCLC cell lines that exhibit a range of non-adherent to adherent phenotypes was evaluated, and only those with an adherent (non-NE) phenotype expressed MHC and TAP1, and exhibited loss of H3K27 methylation. Furthermore, EZH2, which promotes H3K27 methylation and is highly expressed in NE-SCLC, exhibited a negative correlation with TAP1 in an analysis of all SCLC cell lines from the Cancer Cell Line Encyclopedia (CCLE) database. Thus, transient treatment of NE SCLC cell lines with EZH2-inhibitors resulted in generation of an AXL-positive mesenchymal, non-NE phenotype with restored expression of TAP1 and MHC-I, which was also driven by STING and IFN signaling. Together, these data suggest a model in which EZH2 activity in SCLC maintains a NE, TAP1-low state, whereas EZH2 inhibition promotes AXL expression and upregulation of TAP1, STING, IFN and MHC-I.

In consonance with these findings in human SCLC, mouse non-NE SCLC lines were demonstrated to be uniquely immunogenic, and while they were able to form tumors initially, they were later rejected. Tumors formed from non-NE SCLC tumors were highly infiltrated with multiple immune cell populations, including effector $CD8^+$ T cells and M1 macrophages [108]. TCR clonotyping analyses found that a dominant $CD8^+$ T cell

clone (representing over 10% of tumor infiltrating T cells) had formed among T cells infiltrating non-NE-SCLC tumors, while T cells infiltrating NE-SCLC tumors lacked clonality. Immunodominant T cell clones from non-NE SCLC tumors were found to recognize an antigen restricted to non-NE SCLC cells, including those derived by EZH2 inhibitor treatment of NE SCLC cells. Treatment of NE-SCLC cells with EZH2-inhibitor thus primed response to STING agonism *in vivo*, resulting in T cell recognition and complete rejection of tumors in the majority of mice.

In prostate cancer models, EZH2-inhibition has also recently been shown to activate a dsRNA-STING-IFN signaling pathway, suggesting EZH2 acts similarly to repress expression of STING and dsRNA in prostate cancer [109]. Furthermore, combining EZH2-inhibition with anti-PD1 treatment led to improved anti-tumor responses in murine prostate cancer models [109].

Together, these studies demonstrate that resistance to checkpoint immunotherapy in SCLC and *KRAS-LKB1* mutant NSCLC, despite having a high tumor mutation burden, is due to silencing of STING and MHC-I, thus disabling display of antigens to the immune system and enabling immune escape. EZH2-inhibition alone or in combination with STING-agonists may be particularly effective in NE-SCLC, by uncovering an immunogenic phenotype that has avoided immunoediting. However, the specific antigens that are uncovered by EZH2-inhibition remain to be determined. These may be endogenous retroviral genes, oncofetal-derived antigens or mutated proteins [46, 110].

Imaging the Tumor Microenvironment and Metabolism

Imaging of the Labile Iron Pool to Understand its Potential as a Therapeutic Target—The labile iron pool (LIP) refers to a pool of redox active chelatable ferrous iron (Fe^{2+}), which is short lived and an essential intermediate in iron homeostasis [111]. The LIP is essential for various metabolic processes including heme biosynthesis and the electron transport chain. As a short-lived metabolic intermediate, the LIP remains poorly understood, but may represent an actionable therapeutic target in cancers [112, 113]. One fundamental limitation in the understanding of the LIP has been the inability to accurately measure it in living organisms, due to its transient nature. For example, the LIP may not be measured using invasive procedures like mass spectrometry, since Fe^{2+} rapidly oxidizes to ferrous iron outside cells. Therefore, these measurements have traditionally been accomplished using fluorescent dyes in cell culture [114]. Recently, a novel molecular imaging tool to study Fe^{2+} in living organisms was developed [115, 116]. Based on the trioxolane antimalarial drug artemisinin, this agent, called ^{18}F-TRX, is a reactivity based probe which decomposes upon exposure to intracellular Fe^{2+} to form a reactive intermediate which binds irreversibly to cellular protein. This agent has been utilized for positron emission tomography (PET) imaging in preclinical models, with increased tissue uptake correlated with the presence of increased Fe^{2+}. Encouragingly, in cancer models, including the prostate cancer cell line PC3, the uptake of the probe increased over time, and moreover the therapeutic effect of Fe^{2+} directed treatments was correlated with uptake of the PET probe [116]. These early proof of principle PET studies demonstrate the ability to measure LIP in prostate cancer, as well as a potential role for subsequent iron directed therapy.

Hyperpolarized ^{13}C as a Biomarker in Prostate Cancer Disease Progression—One of the hallmarks of cancer is altered metabolism, a property which has been exploited for a variety of therapy and imaging methods. In recent years, hyperpolarized ^{13}C magnetic resonance imaging (MRI) has emerged as a method for imaging this altered metabolism [117]. In this method, an isotopically enriched ^{13}C compound, most commonly [1-^{13}C]pyruvate, can be polarized to increase its signal for MRI. In cancer, the metabolic flux of [1-^{13}C]pyruvate is directed toward lactic acid, in the classic Warburg effect. Initially translated into the clinic at UCSF in men with prostate cancer [118], this method has since expanded to a variety of medical centers, including Memorial Sloan Kettering Cancer Center, for study of cancer and other human diseases. One important finding, initially described in animal models, is that the increased production of lactic acid correlates with high grade disease in localized prostate cancer. This finding was recently recapitulated in men with prostate cancer [119]. Interestingly, the conversion of pyruvate to lactic acid was found to be correlated with the transporter, monocarboxylate transporter 1, as well as loss of PTEN. This method has also been applied in metastatic disease [120], again recapitulating the finding of high conversion of pyruvate to lactate. While the initial groundwork suggests that this is a highly promising method for staging and assessing response to treatment in prostate cancer, future important questions include comparison with other imaging methods such as PSMA PET, or if hyperpolarized MRI imaging biomarkers can be used to correlate with the presence of aggressive phenotypes such as castration resistant or neuroendocrine prostate cancer.

Imaging of Response to Immunotherapy—One area of unmet clinical need in cancer imaging has been the development of robust metrics for imaging responses to immunotherapy [121, 122]. In the development of novel molecular imaging agents for immunotherapy, a variety of targets could be considered. Among these, CD8 T-lymphocytes have demonstrated potential, with preclinical and preliminary clinical studies demonstrating promise [123]. Specifically, small antibody formats targeting CD8 with the diabody ^{89}Zr-DFO-169 (mouse-specific) or ^{89}Zr-DFO-IAB22M2C minibody (human-specific) have demonstrated promise for imaging T-cells using PET in preclinical studies. More recently, in a clinical study, the ^{89}Zr-DFO-IAB22M2C minibody demonstrated favorable kinetics with rapid accumulation in CD8-rich tissues [124]. An ongoing area of interest is to see if these novel molecular imaging agents can detect immune responses in the context of patients undergoing immunotherapy. In principle, these novel molecular imaging agents could be used to help predict response to immunotherapy, or to enable rapid response assessment.

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