TARGETED DRUG THERAPY

Dickkopf-1 Can Lead to Immune Evasion in Metastatic Castration-Resistant Prostate Cancer

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PURPOSE Metastatic castration-resistant prostate cancer (mCRPC) with low androgen receptor (AR) and without neuroendocrine signaling, termed double-negative prostate cancer (DNPC), is increasingly prevalent in patients treated with AR signaling inhibitors and is in need of new biomarkers and therapeutic targets.

METHODS Candidate genes enriched in DNPC were determined using differential gene expression analysis of discovery and validation cohorts of mCRPC biopsies. Laboratory studies were carried out in human mCRPC organoid cultures, prostate cancer (PCa) cell lines, and mouse xenograft models. Epigenetic studies were carried out in a rapid autopsy cohort.

RESULTS Dickkopf-1 (DKK1) expression is increased in DNPC relative to prostate-specific antigen (PSA)–expressing mCRPC in the Stand Up to Cancer/Prostate Cancer Foundation discovery cohort (11.2 *v* 0.28 reads per kilobase per million mapped reads; $q < 0.05$; n = 117) and in the University of Washington/Fred Hutchinson Cancer Research Center cohort (9.2 *v* 0.99 fragments per kilobase of transcript per million mapped reads; $P < .0001$). DKK1 expression can be regulated by activated Wnt signaling in vitro and correlates with activating canonical Wnt signaling mutations and low PSA mRNA in mCRPC biopsies ($P < .05$). *DKK1* hypomethylation was associated with increased DKK1 mRNA expression (Pearson $r = -0.66$; $P < .0001$) in a rapid autopsy cohort (n = 7). DKK1-high mCRPC biopsies are infiltrated with significantly higher numbers of quiescent natural killer (NK) cells ($P < .005$) and lower numbers of activated NK cells ($P < .0005$). Growth inhibition of the human PCa model PC3 by the anti-DKK1 monoclonal antibody DKN-01 depends on the presence of NK cells in a severe combined immunodeficient xenograft mouse model.

CONCLUSION These results support DKK1 as a contributor to the immunosuppressive tumor microenvironment of DNPC. These data have provided the rationale for a clinical trial targeting DKK1 in mCRPC (ClinicalTrials.gov identifier: NCT03837353).

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INTRODUCTION

Androgen receptor (AR) signaling, the dominant oncogenic pathway in metastatic castration-resistant prostate cancer (mCRPC), can be pharmacologically targeted by next-generation anti-androgens and inhibitors of androgen biosynthesis, such as enzalutamide and abiraterone. Resistance to AR-directed therapy through an AR-indifferent escape mechanism has been associated with loss of the tumor suppressors *TP53*, *RB1*, and *PTEN*[1-3]; histologic transdifferentiation into neuroendocrine prostate cancer (NEPC); and low prostate-specific antigen (PSA) expression. Recently, a non-neuroendocrine subtype of advanced prostate cancer with low PSA expression, termed double-negative prostate cancer (DNPC), has been reported by the University of Washington/Fred Hutchinson Cancer Research Center (UW/FHCRC).[4] Preclinical models and analysis of biopsy specimens support the importance of autocrine and paracrine fibroblast growth factor and mitogen-activated protein kinase signaling in bypassing the requirement for AR signaling in these cancers.[4] Recent work using [^{18}F] dihydrotestosterone (FDHT) positron emission tomography (PET) imaging of patients with mCRPC has shown that 49.6% of a cohort of 133 patients with mCRPC have at least one metastatic site that is [^{18}F] fluorodeoxyglucose (FDG) avid but FDHT negative and that these lesions tend to be PSA negative by immunohistochemistry and portend a poor prognosis.[5] Controversy remains about the appropriate histopathologic classification and nomenclature of treatment-resistant prostate cancers largely because of the paucity of available metastatic tissue.

Genetic studies have found multiple oncogenic pathways to be altered in mCRPC, including the Wnt signaling pathway.[6] Canonical Wnt signaling revolves

ASSOCIATED CONTENT

Appendix

Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

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CONTEXT

Key Objective

We sought to define targetable drivers in double-negative prostate cancer (DNPC), an emerging subtype of metastatic castration-resistant prostate cancer (mCRPC).

Knowledge Generated

We identified upregulation of Dickkopf-1 (DKK1) in DNPC tumor samples. We demonstrated that DKK1 expression in prostate cancer correlates with DKK1 promoter hypomethylation, a mesenchymal transcriptional signature, and that DKK1 can be regulated by canonical Wnt signaling. DKK1-expressing mCRPC biopsies are infiltrated by quiescent rather than active natural killer (NK) cells, elevated M2 macrophages, and diminished CD8+ T cells, consistent with immune evasion. Therapeutic DKK1 blockade slows the growth of a prostate cancer mouse xenograft model in an NK-cell–dependent manner.

Relevance

Our data point to DKK1 as a potential therapeutic target for DNPC and provide the rationale for an active clinical trial for DKK1-expressing mCRPC.

around the regulation of β-catenin (gene name *CTNNB1*).[7] Mutations deleterious to APC or stabilizing in β-catenin lead to constitutive activation of the pathway. In addition, mutation or deletion of ubiquitin ligases ZNRF3 and RNF43, along with amplification of RSPO2, amplify the Wnt signal through increases in Wnt/Frizzled receptor stability. Multiple lines of evidence have suggested that Wnt inhibition might be an effective treatment of mCRPC.[8-10] Dickkopf-1 (DKK1) is a secreted protein characterized in mesenchymal tissues to be a Wnt antagonist that binds the LRP coreceptor, leading to diminished signaling through the Wnt/Frizzled receptor complex.[11] While initially characterized as a tumor suppressor because of its Wnt inhibitory effects, DKK1 was discovered to promote tumor growth and invasion in multiple forms of cancer, including prostate cancer,[12] possibly through activation of phosphatidylinositol 3-kinase/AKT signaling through its interaction with CKAP4.[13] Nevertheless, the mechanism for DKK1's effect on tumor growth is likely context specific, possibly differing even within individual types of cancer.

Given the finding that there exists a substantial portion of mCRPC tumors with low PSA and low AR expression, we sought to determine whether there are biomarkers and therapeutic targets that align with low rather than high AR activity. Here, we report our efforts to identify candidate genes enriched in prostate adenocarcinomas with low PSA. We specifically excluded histologically confirmed NEPCs from this analysis given the extensive work that has already been published on these tumors. We analyzed mCRPC biopsies collected as part of the Stand Up to Cancer/Prostate Cancer Foundation (SU2C/PCF) cohort study in conjunction with the biopsy/autopsy cohort collected at UW/FHCRC. Our analysis was further validated in the recently derived human mCRPC organoid culture models,[14] in several clinically annotated cohorts of patients with mCRPC, and in preclinical models.

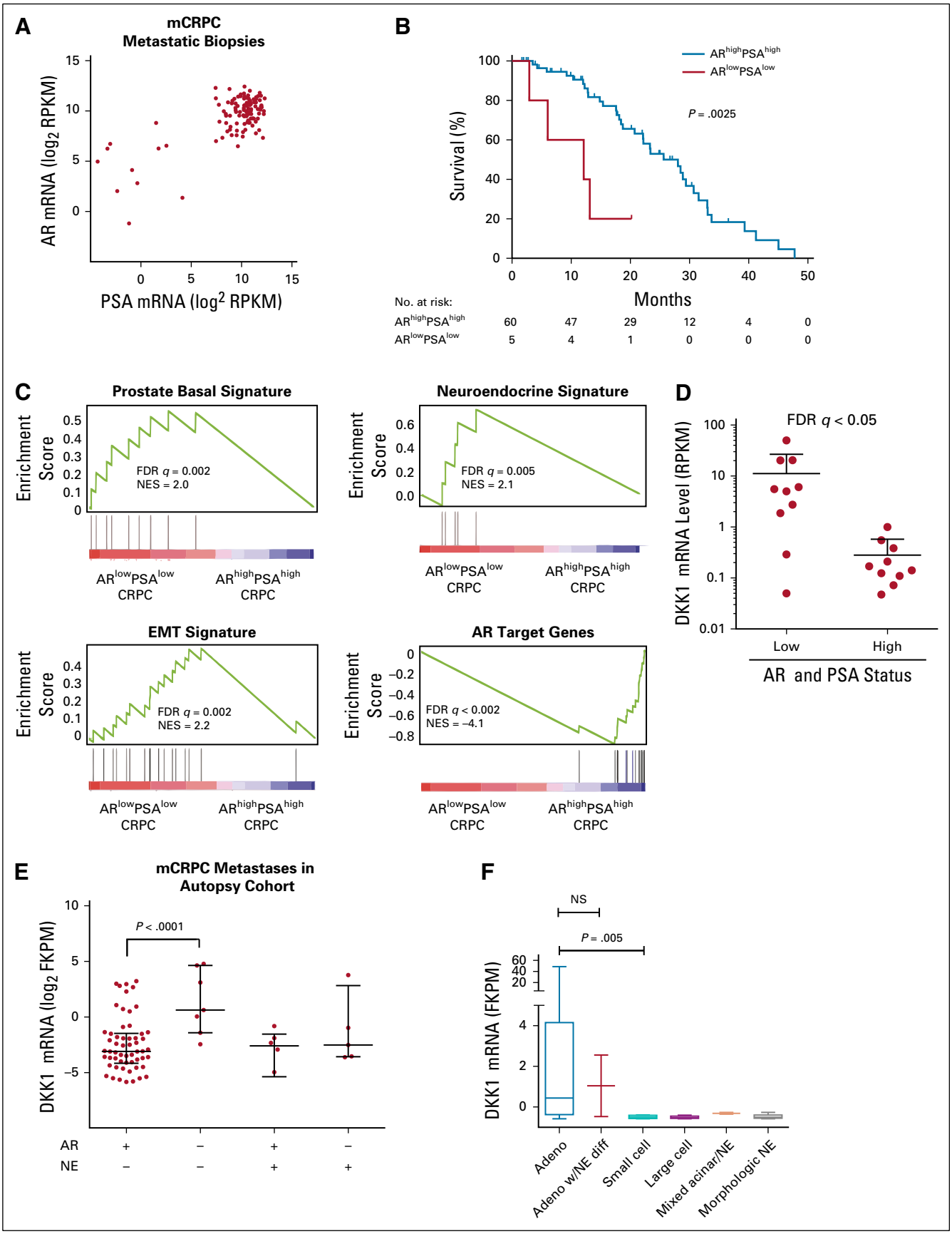
METHODS

RNA sequencing data from metastatic biopsies from the 117-patient SU2C/PCF cohort[6] were included in the differential gene expression analysis. Cases with histologic evidence of small-cell or large-cell carcinoma were excluded. Histopathology analysis of these cases was reviewed centrally and described previously.[6] $AR^{low}PSA^{low}$ cases were defined as those with both AR and PSA mRNA in the bottom 10th percentile for the cohort. $AR^{high}PSA^{high}$ cases were selected from the remainder of the cohort and chosen to best match features of AR^{low} cases by biopsy site, sequencing facility, and tumor content (Data Supplement). Kaplan-Meier analysis was performed for overall survival of the SU2C/PCF cohort of patients treated with first-line enzalutamide or abiraterone as previously published.[15] Metastatic biopsies with mRNA data (capture assay) were included in the analysis. Specific details about the gene set enrichment analysis (GSEA), UW/FHCRC validation cohort, and Wnt mutational categorizations are provided in the Appendix. Methods for cell culture, tumor models, DKK1 enzyme-linked immunosorbent assay, and DKK1 methylation analysis also are provided in the Appendix.

RESULTS

DKK1 and PSA Expression Are Inversely Correlated in mCRPC

Of the published SU2C/PCF cohort of mCRPC biopsies,[6,15] we focused on the cases in which both AR and PSA mRNA expression were in the bottom 10th percentile for the cohort on the basis of RNA sequencing analysis (Fig 1A). We excluded cases of small-cell or large-cell prostate cancer or with inadequate tumor content. Of the patients treated with first-line novel hormonal agents abiraterone or enzalutamide, those with $AR^{low}PSA^{low}$ disease demonstrated a significantly shorter overall survival (12.1 *v* 28.1 months; $P = .0025$; Fig 1B). This result supports data that show that


A
mCRPC
Metastatic Biopsies
AR mRNA (log2 RPKM)
PSA mRNA (log2 RPKM)
B
ARhighPSAhigh
ARlowPSAlow
P = .0025
Survival (%)
Months
No. at risk:
ARhighPSAhigh 60 47 29 12 4 0
ARlowPSAlow 5 4 1 0 0 0
C
Prostate Basal Signature
FDR q = 0.002
NES = 2.0
Neuroendocrine Signature
FDR q = 0.005
NES = 2.1
EMT Signature
FDR q = 0.002
NES = 2.2
AR Target Genes
FDR q < 0.002
NES = –4.1
Enrichment Score
ARlowPSAlow CRPC
ARhighPSAhigh CRPC
D
FDR q < 0.05
DKK1 mRNA Level (RPKM)
Low
High
AR and PSA Status
E
mCRPC Metastases in Autopsy Cohort
P < .0001
DKK1 mRNA (log2 FKPM)
AR
NE
F
NS
P = .005
DKK1 mRNA (FKPM)
Adeno
Adeno w/NE diff
Small cell
Large cell
Mixed acinar/NE
Morphologic NE

patients with PSA^{low}-secreting tumors progressed faster on AR therapy than chemotherapy and had a shorter overall survival.[16]

We carried out a differential gene expression analysis that compared 10 AR^{low}PSA^{low} tumors with 10 AR^{high}PSA^{high} tumors, the latter selected to best match the organ biopsy site and the institution at which the biopsy was processed (Data Supplement). GSEA of the set of differentially expressed genes demonstrated enrichment of nonluminal lineage programs, including basal-type,[17] neuroendocrine,[3] and mesenchymal enrichment (Fig 1C), in the AR^{low}PSA^{low} compared with the AR^{high}PSA^{high} cases. Of note, of the neuroendocrine-associated genes, *N-Myc* was significantly upregulated (4.4 *v* 0.3 reads per kilobase per million mapped reads [RPKM]; Mann-Whitney test $P < .005$), Aurora kinase A was not significantly upregulated (9.1 *v* 8.6 RPKM; Mann-Whitney test $P = .91$), and DLL3 was of borderline significance (0.6 *v* 0.23; Mann-Whitney test $P = .08$) in AR^{low}PSA^{low} compared with AR^{high}PSA^{high} tumors. Genomic profiling of AR^{low}PSA^{low} biopsies showed a higher prevalence of altered *RB1* (50% *v* 6%; Fisher's exact test $P < .0001$) and wild-type AR (0% *v* 65%; $P < .001$).

Of the most differentially expressed genes in the AR^{low}PSA^{low} biopsies, our focus on druggable and secreted gene products led us to DKK1, a mesenchymal lineage gene and target of canonical Wnt signaling.[18] DKK1 is upregulated in the AR^{low}PSA^{low} subset in the SU2C/PCF cohort (11.2 *v* 0.28 RPKM, $q < 0.05$; Fig 1D). Since the original analysis, the SU2C/PCF cohort has been expanded to 212 patients with full sequencing data, including eight additional AR^{low}PSA^{low} cases. DKK1 expression remains significantly increased ($P = .0096$) in the expanded cohort. There is also a significant anticorrelation between DKK1 and PSA mRNA levels (Spearman $r = –0.27$; $P < .0001$) and between DKK1 mRNA and AR signature score ($r = –0.47$; $P < .0001$). There was no significant difference in DKK1 levels in abiraterone- or enzalutamide-treated patients versus treatment-naive patients (Appendix Fig A1A). DKK1 is also elevated in non-neuroendocrine AR^{low}PSA^{low} mCRPC relative to AR^{high}PSA^{low} mCRPC (9.2 *v* 0.99 fragments per kilobase of transcript per million mapped reads [FPKM]; $P < .0001$) in the UW/FHCRC cohort, which consisted of 77 metastatic biopsies annotated by AR and neuroendocrine gene expression status[4] (Fig 1E). By contrast, analysis of publicly available data shows that DKK1 is not expressed in treatment-related small-cell prostate cancers ($P = .005$; Fig 1F), and this is consistent with a prior published analysis of these data.[3] Metastatic prostate cancer grown in organoid culture also recapitulates this pattern (Appendix Fig A1B) because DKK1 mRNA was increased in non-neuroendocrine AR^{low} prostate cancer organoids MSK-PCa1 (33.1 FPKM) and MSK-PCa6 (8.1 FPKM) compared with the AR^{high} models MSK-PCa2 (0.88 FPKM) and MSK-PCa-7 (0 FPKM). The AR^{low} neuroendocrine organoid model MSK-PCa4 also showed low-level DKK1 expression (0.20 FPKM).

Circulating DKK1 Levels Are Increased in mCRPC With Low FDHT Uptake

After confirming that DKK1 is secreted in vitro (Appendix Fig A1C), we tested the hypothesis that circulating DKK1 might predominate in patients with mCRPC and a relatively low serum PSA. To address this, serum DKK1 and PSA levels were measured in a cohort of patients who had undergone sequential FDHT and FDG PET imaging.[5] Biopsy of a subset of these lesions confirmed that immunohistochemical PSA staining strongly correlated with FDHT uptake and that lesions without FDHT uptake tended to lack PSA immunostaining. The presence of these lesions also prognosticated poor outcome.[5] Patients with higher tumoral FDHT uptake had higher serum PSA levels (Fig 2A; Appendix Fig A1D). By contrast, and in further support of the hypothesis that AR^{low}PSA^{low} have increased DKK1 expression, patients with higher tumoral FDHT trended toward having lower serum DKK1 levels (Fig 2B; Appendix Fig A1E). To better determine the association of prostate cancer disease state with circulating DKK1, we opted for testing DKK1 in plasma, rather than serum, on the basis of a previous report that showed that high levels of DKK1 are released from platelets during preparation of serum.[19] Indeed, we found that plasma DKK1 was significantly elevated in patients with mCRPC compared with healthy control men (1,880 *v* 665 pg/mL; $P < .0001$), whereas serum DKK1 level was no different between the two groups (Fig 2C; Appendix Fig A1F). Of the 23 patients with mCRPC analyzed, 10 (43%) had plasma DKK1 levels higher than the maximum level measured in the 50 healthy control men analyzed. Finally, plasma DKK1 was higher in mCRPC than in metastatic hormone-sensitive prostate cancer (mHSPC) (1,910 *v* 519 pg/mL; $P < .05$; Fig 2D). Changes in disease burden were not quantified for these patients and cannot be excluded as an explanation for the increase in plasma DKK1 levels in mCRPC compared with mHSPC.

FIG 1. Elevated Dickkopf-1 (DKK1) expression in lethal non-neuroendocrine low–androgen receptor (AR^{low}) and low–prostate-specific antigen (PSA^{low}) metastatic castration-resistant prostate cancer (mCRPC). (A) AR and PSA mRNA levels in 117 mCRPC biopsies. (B) Kaplan-Meier survival analysis of patients with AR^{low}PSA^{low} biopsies (n = 5) compared with the remainder of the cohort (n = 60). (C) Gene set enrichment analysis of differentially expressed AR^{low}PSA^{low} and AR^{high}PSA^{high} using references gene sets. (D) DKK1 mRNA levels in AR^{low}PSA^{low} (n = 10) and AR PSA^{high} (n = 10). (E) DKK1 mRNA levels in mCRPC biopsies. (F) DKK1 mRNA levels derived from publicly available RNA sequencing data from a Weill-Cornell Medical College mCRPC cohort.[3] Adeno, adenocarcinoma; Adeno w/ NE diff, adenocarcinoma with neuroendocrine differentiation; EMT, epithelial-mesenchymal transition; FDR, false discovery rate; FPKM, fragments per kilobase of transcript per million mapped reads; NE, neuroendocrine; NES, normalized enrichment score; NS, not significant; RPKM, reads per kilobase per million mapped reads.

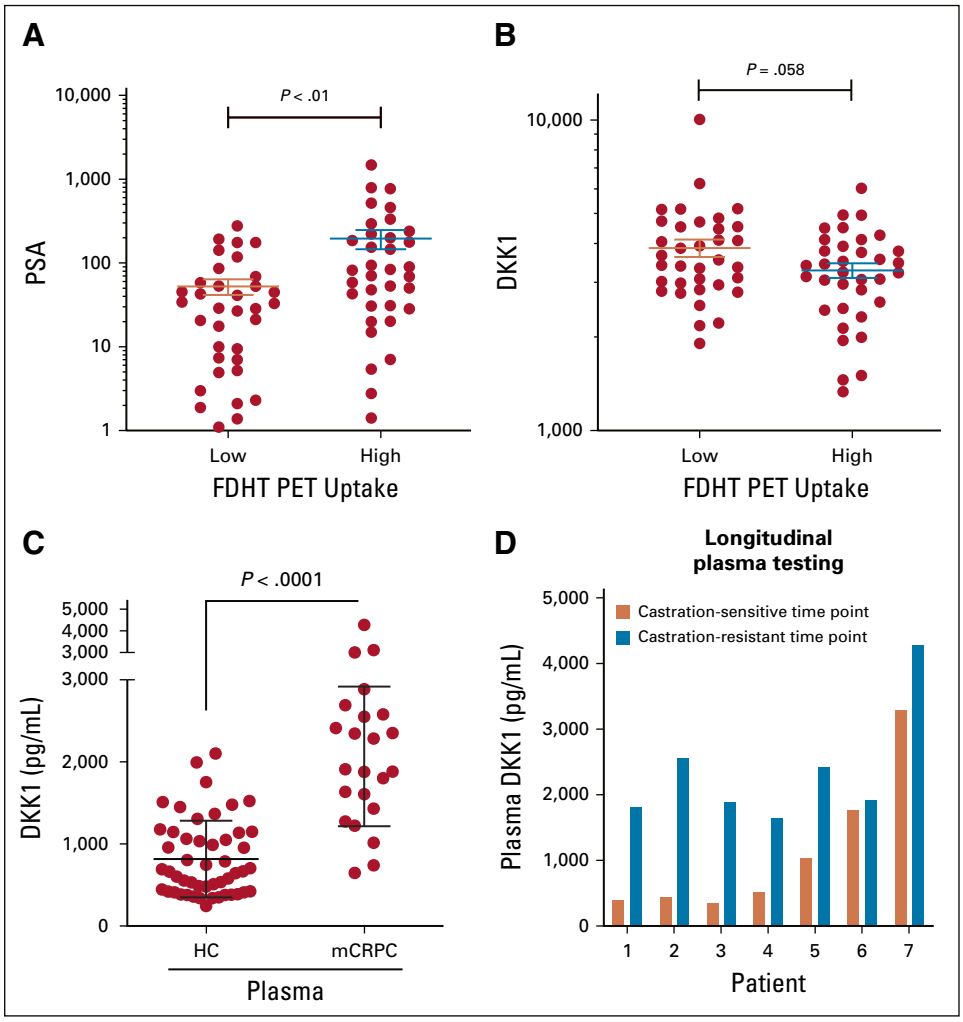


FIG 2. Circulating Dickkopf-1 (DKK1) levels are elevated in patients with metastatic castration-resistant prostate cancer (mCRPC) and inversely correlate with tumoral [^{18}F]dihydrotestosterone (FDHT) uptake. (A) Serum prostate-specific antigen (PSA) and (B) serum DKK1 quantitated in patients with mCRPC who underwent FDHT positron emission tomography (PET) imaging. Patients are grouped on the basis of total FDHT uptake of all metastatic lesions above (high) and below (low) the median for the cohort. (C) Plasma DKK1 protein quantitated in healthy control (HC) men and patients with mCRPC. (D) Plasma DKK1 protein quantitated in treatment-naive patients with metastatic castrate-sensitive prostate cancer and matched samples from onset of mCRPC.

Activated Canonical Wnt Signaling Can Regulate DKK1 in mCRPC

The *DKK1* gene is amplified in a small percentage of patients with mCRPC, but amplification did not correlate with increased DKK1 expression, which suggests more complex mechanisms of regulation (Appendix Figs A1G and A1H). To test whether alterations in Wnt signaling can modulate DKK1 expression in prostate cancer, we activated canonical Wnt with overexpression of mutant stabilized β-catenin or knockdown of APC in LNCaP-abl cells, a preclinical model of CRPC. DKK1 mRNA levels were significantly increased with both methods, along with the well-established Wnt target gene AXIN2 (Fig 3A). DKK1 mRNA increased 12.5-fold with stabilized β-catenin and 2.2-fold with shAPC, while AXIN2 mRNA had the same trend, increasing 21.8 and 2.4-fold. In addition, treatment of the AR-negative human prostate cancer cell line PC3 as well as LNCaP-abl cells with cyclic 13, a previously described novel specific inhibitor of the β-catenin/TCF interaction,[20] led to a reproducible and substantial reduction in the levels of DKK1 (Fig 3B). Collectively, these data support activated canonical Wnt signaling as an upstream regulator of DKK1 in prostate cancer. Surprisingly, analysis of mCRPC biopsies in the SU2C/PCF cohort revealed that activating Wnt pathway mutations correlate with elevated DKK1 expression within PSA^{low} mCRPC ($P <$ .05) but not within PSA-expressing mCRPC (Fig 3C). Expression of the Wnt target gene AXIN2 is similarly further increased in the Wnt mutant, PSA^{low} tumors (Appendix Fig A1I) relative to the Wnt mutant, PSA^{high} tumors. The finding that the PSA^{low} state is permissive of increased Wnt output is consistent with a previous report that showed that downregulation of AR signaling leads to increased transcriptional activity of β-catenin at TCF4 binding sites.[21] No specific Wnt mutation is over-represented in the PSA^{low}, Wnt mutant cases, which include an APC deletion, an RSPO2 amplification, a ZNRF3 deletion, and two stabilizing CTNNB1 missense mutations.

Hypomethylation of the DKK1 Promoter Correlates With DKK1 Expression in mCRPC

Prior literature supports the importance of promoter methylation in the regulation of DKK1 expression.[22] To test whether epigenetic alterations that involve the *DKK1* locus are associated with increased DKK1 expression, we

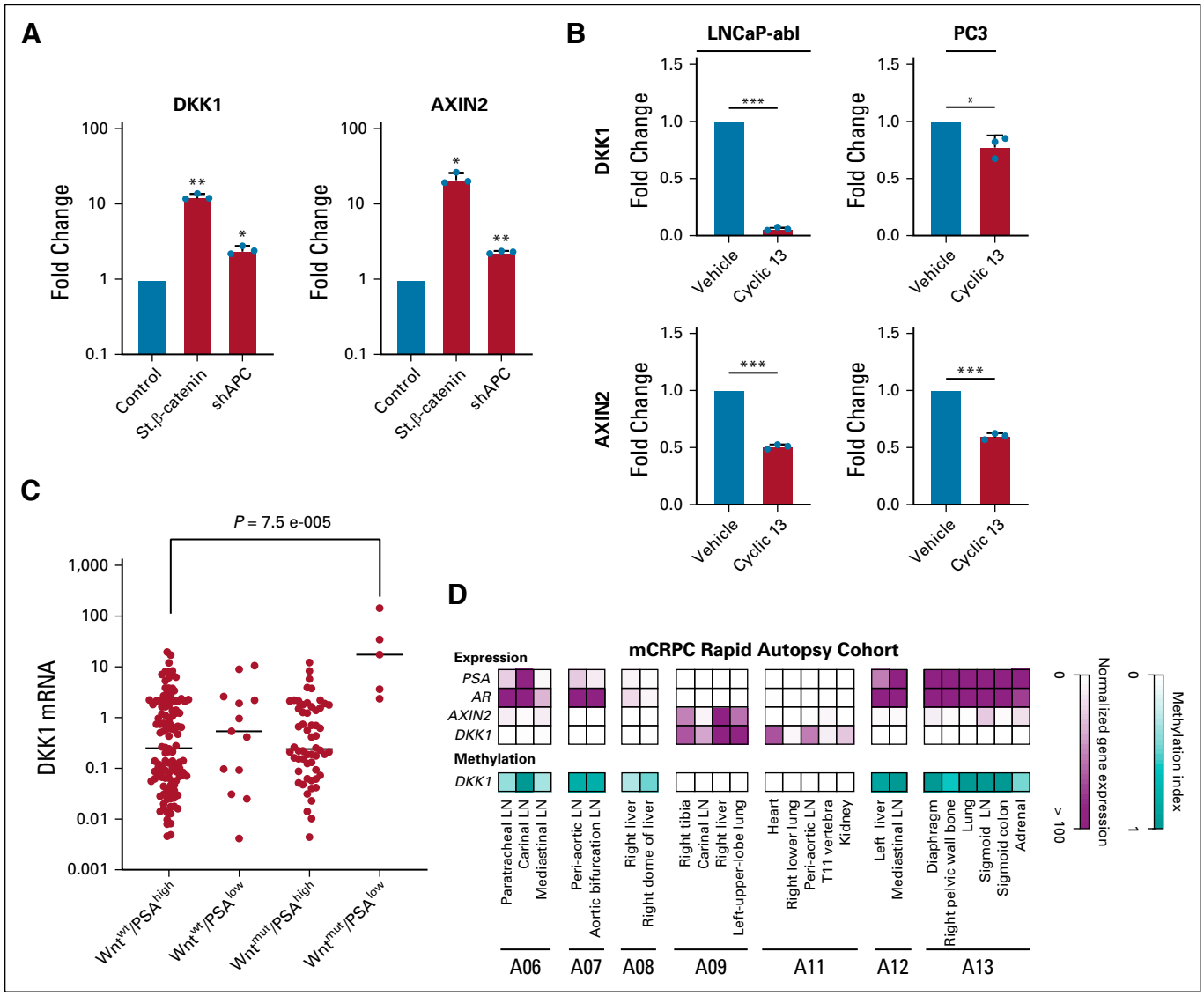


FIG 3. Dickkopf-1 (DKK1) is modulated by canonical Wnt signaling and hypomethylation in advanced prostate cancer. (A) Quantitative real-time polymerase chain reaction (RT-qPCR) of mRNA[AE1] from LNCaP-abl cells with either induced stabilized (St.; hot spot mutant [mut]) b-catenin or APC knockdown. (B) RT-qPCR from LNCaP-abl and PC3 cells treated with Wnt inhibitor cyclic 13 for 24 hours at 10 mM. (C) mRNA levels of DKK1 in the Stand Up to Cancer/Prostate Cancer Foundation cohort of tumor samples. Wnt^{mut} tumors defined as pathogenic mutations in either APC, CTNNB1, RNF43, ZNRF3, or RSPO2. (D) Integrated DNA methylation and mRNA expression analyses of 25 anatomically distinct metastatic deposits from seven patients with metastatic castration-resistant prostate cancer (mCRPC) procured by rapid autopsy revealed hypomethylation of the DKK1 locus with associated high expression of DKK1 mRNA in cases with low androgen receptor (AR^{low}) and low prostate-specific antigen (PSA^{low}) expression. Heat map codes: methylation: blue, dense methylation; white, no methylation; mRNA expression: red, high expression; white, low expression. Statistical differences for RT-qPCR were calculated by unpaired *t* tests. (*)$P < .05$, (**)$P < .01$, (***)$P < .001$, where each point represents an individual biologic replicate. LN, lymph node; wt, wild type. [AE1]In Figure 3 legend, please confirm that the abbreviation RT-qPCR is expanded correctly.

evaluated the methylation status of a CpG island in proximity to the transcriptional start site of *DKK1* in 25 anatomically distinct metastatic deposits from seven patients with mCRPC (Fig 3D). All lesions showed morphologic features of prostatic adenocarcinoma and were negative for neuroendocrine markers. Five of the seven that were positive for AR and PSA expression showed *DKK1* hypermethylation in all metastatic sites tested. Two (A09, A11) showed an absence of CpG methylation of *DKK1*. *DKK1* hypomethylation was associated with increased DKK1 mRNA expression (Pearson $r = -0.66$; $P < .0001$), which provided evidence that DKK1 expression is regulated at the level of CpG methylation in mCRPC. *DKK1* hypomethylation was highly conserved between different metastases from a given patient and occurred exclusively in cases characterized by AR^{low} and PSA^{low} expression. Of note, one out

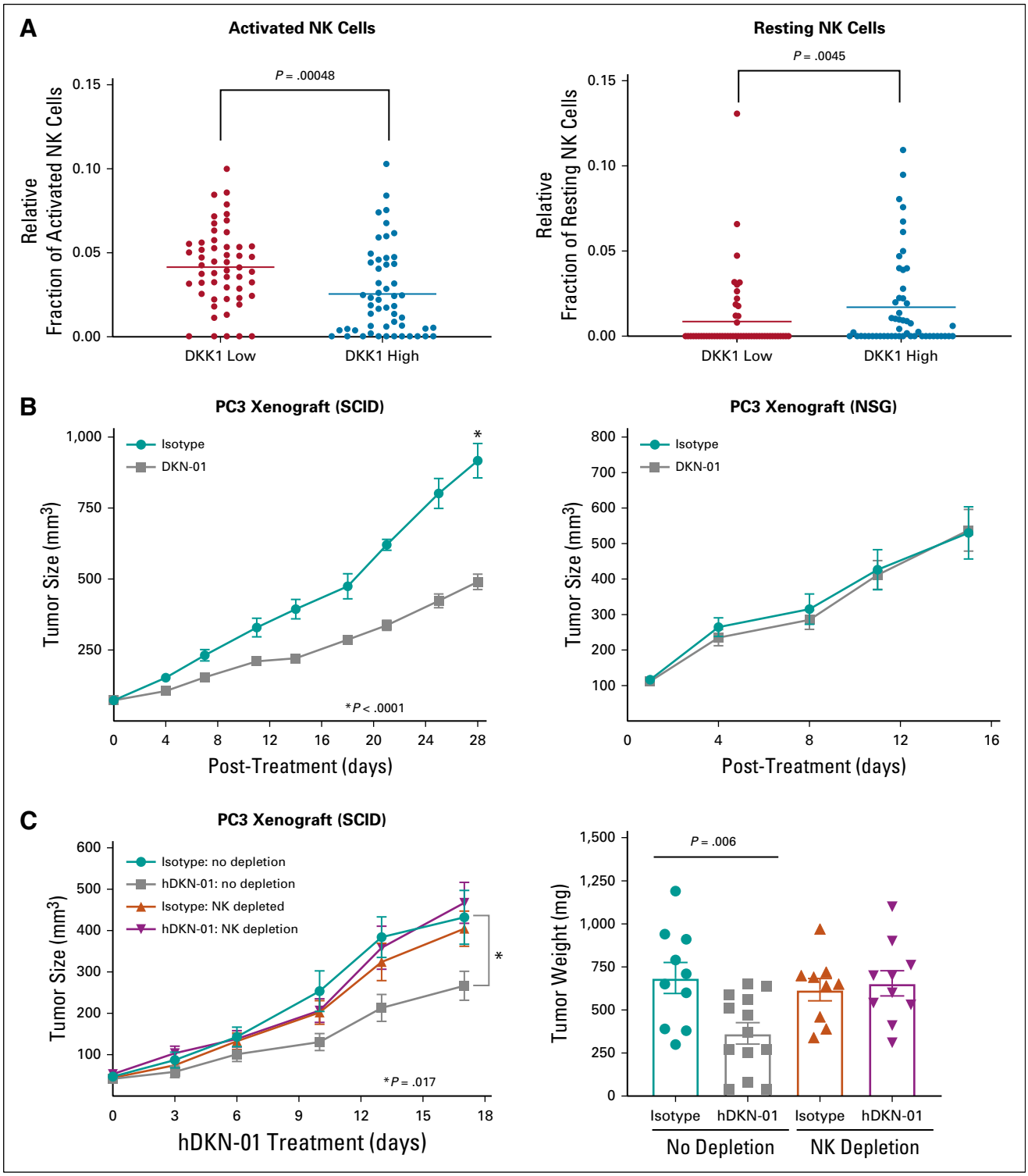


FIG 4. Dickkopf-1 (DKK1) can contribute to immune evasion through modulation of natural killer (NK) cell activity. (A) CIBERSORT2 immune cell composition analysis of the Stand Up to Cancer/Prostate Cancer Foundation metastatic castration-resistant prostate cancer cohort (n = 212) was carried out. DKK1 low and high groupings represent the bottom and top quartiles of DKK1 mRNA expression. (B) and (C) Mean tumor volumes and SEM are plotted. *P* values from two-way repeated-measures analysis of variance with Sidak's multiple comparisons. Representative data from one of two independent experiments are shown. SCID, severe combined immunodeficient.

of the two patients with high DKK1 expression also expressed high levels of AXIN2 mRNA, which supports the co-occurrence of activated Wnt signaling with DKK1 upregulation in mCRPC. Collectively, these findings suggest that *DKK1* hypomethylation might be an important mechanism responsible for DKK1 expression in $AR^{low}PSA^{low}$ CRPC.

DKK1 Blockade Can Slow Tumor Growth in an NK-Cell Dependent Manner

Prior observations have independently linked DKK1[18,23] and activated canonical Wnt signaling with immune evasion.[24,25] On the basis of these prior reports, we tested whether DKK1 correlates with immune evasion in mCRPC. CIBERSORT[26] immune cell composition analysis of the SU2C/PCF mCRPC cohort (n = 212) was carried out, and DKK1 levels were correlated with immune signatures of immunosuppression, including increased M2 macrophages ($P < .05$), decreased CD8 T cells ($P = .01$), higher levels of quiescent NK cells, and lower levels of activated NK cells as well as other immune lineages (Data Supplement; Fig 4A). To test the contribution of NK cells to DKK1-mediated tumor growth, we used the DKK1-expressing human prostate cancer cell line PC3. DKK1 blockade, using the DKK1-neutralizing monoclonal antibody DKN-01, led to a significant reduction in tumor growth in the NK-cell proficient severe combined immunodeficient (SCID) mouse model. By contrast, DKK1 blockade had no effect on PC3 tumor growth in the NSG mouse strain, which lacked NK cells (Fig 4B). These results are consistent with a role for NK cells with a DKK1-targeting therapy in prostate cancer. To more specifically interrogate the impact of NK cells, we used the NK-cell–depleting antibody anti-asialo GM1 in the PC3 SCID xenograft model. We found that in NK-depleted mice, DKK1 blockade was no longer effective at inhibiting tumor growth (isotype *v* hDKN-01 with no depletion, $P = .0017$; isotype *v* hDKN-01 with NK-cell depletion, $P = .347$; Fig 4C). There was also a significant difference in tumor weights (isotype *v* hDKN-01 with no depletion, $P = .006$; isotype *v* hDKN-01 with NK-cell depletion, $P = .711$). Collectively, these data support a requirement for NK cells in the antitumor effect of DKK1 depletion.

DISCUSSION

We report, to our knowledge for the first time, that DKK1 is upregulated in a subset of DNPC. Further validation of DNPC as a clinically distinct subtype of CRPC is needed. Nevertheless, multiple lines of evidence support the inverse relationship between AR signaling and DKK1 expression. DNPC biopsy specimens and DNPC organoid models tend to exhibit elevated DKK1 expression, and the highest levels of circulating DKK1 are found in patients with low tumoral FDHT uptake on PET imaging. DNPC tumors with activated Wnt signaling seem to express even higher levels of DKK1.

Previous analyses have found that serum DKK1 at the time of localized prostate cancer diagnosis[27] and tumoral DKK1 expression in metastatic prostate cancer tissue[28] both prognosticate poor overall survival. We hypothesize that DKK1 expression could be a circulating biomarker that could reflect the presence of mCRPC with low PSA. This is of particular importance given the recent work demonstrating profound intrapatient heterogeneity of AR signaling.[5] Low FDHT uptake on a PET scan coupled with elevated circulating DKK1 could enable early detection of otherwise occult mCRPC with low PSA. Future validation of plasma DKK1 levels in patients with DNPC will be important for confirming its prognostic value as a biomarker.

Activated canonical Wnt signaling has been reported to mediate immunosuppression and resistance to checkpoint blockade in multiple cancer types.[24,29] DKK1 has been shown to promote tumor growth in preclinical models through modulation of NK cells and myeloid-derived suppressor cells.[18,23] Here we provide, to our knowledge, the first evidence that DKK1 can be regulated by Wnt signaling in CRPC and that DKK1 expression in human mCRPC biopsies correlates with deficient NK-cell activity. NK-cell recruitment has been shown to be Wnt dependent, which suggests that DKK1 could be having a direct effect on NK cells.[30] Alternatively, DKK1 has also been shown to decrease NK-cell–activating ligands in the tumor microenvironment (TME) of breast and lung cancers, a potential indirect mechanism.[23] Whether through direct or indirect signaling, our xenograft studies demonstrate that the effect of DKK1 antibodies to slow prostate cancer progression can be NK-cell dependent.

We hypothesize that DKK1 may be a key mediator of the immunosuppression in DNPC. mCRPC tumors with high DKK1 expression exhibit a $CD8^+$ T-cell–poor TME infiltrated with immature M0 macrophages, M2 macrophages, neutrophils, and quiescent NK cells. Studies on the effects of DKK1 on other arms of the immune system are ongoing. In particular, syngeneic models will be necessary to test DKK1's effect on T cells and B cells.

Here, we provide evidence that the DKK1 promoter is epigenetically silenced through CpG methylation. Of note, we found that mCRPC tumors with low PSA and elevated DKK1 show evidence of DKK1 promoter hypomethylation. Whether there is a link between reduced AR activity and promoter hypomethylation is currently unknown, but this result supports DNA methylation as a likely mechanism that governs DKK1 expression in mCRPC. A further implication of this result is that elevated DKK1 could be a resistance mechanism to therapeutic DNA methyltransferase inhibition, which is currently being tested in clinical trials.

Our findings provided the rationale for an active clinical trial (ClinicalTrials.gov identifier: NCT03837353) testing DKK1 blockade in CRPC. This trial will be a critical platform for

assessing the clinical relevance of our findings. Analysis of DKK1 expression, activated Wnt signaling, and markers of AR activity in pretreatment specimens collected as part of this clinical trial will address whether these biomarkers can predict treatment response. Immunophenotyping of peripheral blood and tumor specimens collected as part of this study will be used to investigate the immunomodulatory effects of therapeutic DKK1 blockade in patients.

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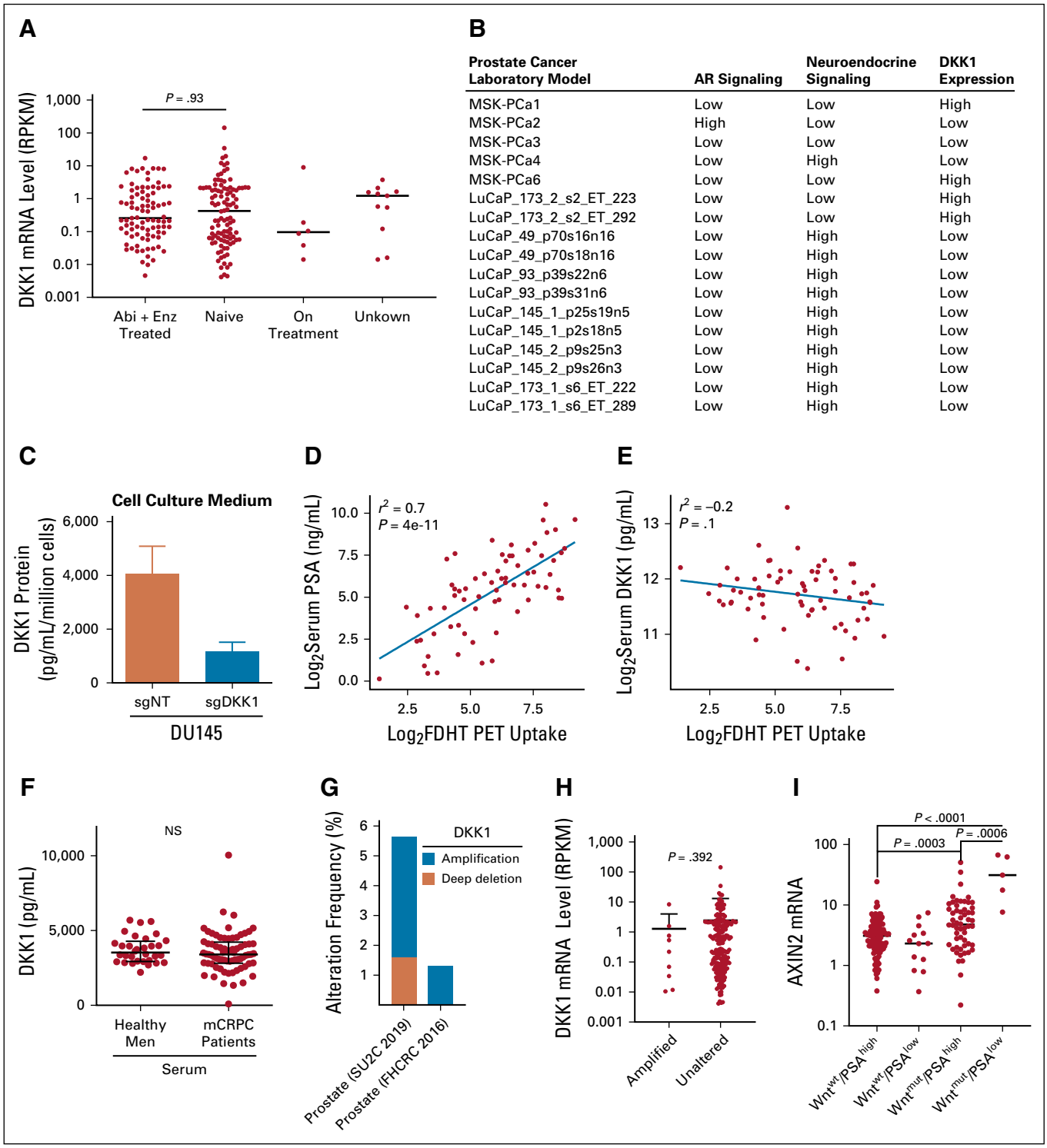

Prostate Cancer Laboratory Model	AR Signaling	Neuroendocrine Signaling	DKK1 Expression
MSK-PCa1	Low	Low	High
MSK-PCa2	High	Low	Low
MSK-PCa3	Low	Low	Low
MSK-PCa4	Low	High	Low
MSK-PCa6	Low	Low	High
LuCaP_173_2_s2_ET_223	Low	Low	High
LuCaP_173_2_s2_ET_292	Low	Low	High
LuCaP_49_p70s16n16	Low	High	Low
LuCaP_49_p70s18n16	Low	High	Low
LuCaP_93_p39s22n6	Low	High	Low
LuCaP_93_p39s31n6	Low	High	Low
LuCaP_145_1_p25s19n5	Low	High	Low
LuCaP_145_1_p2s18n5	Low	High	Low
LuCaP_145_2_p9s25n3	Low	High	Low
LuCaP_145_2_p9s26n3	Low	High	Low
LuCaP_173_1_s6_ET_222	Low	High	Low
LuCaP_173_1_s6_ET_289	Low	High	Low

FIG A1. (A) Comparison of Dickkopf-1 (DKK1) mRNA levels by treatment status the Stand Up to Cancer/Prostate Cancer Foundation (SU2C/PCF) metastatic castration-resistant prostate cancer (mCRPC) cohort. (B) Table of available preclinical models for the study of DKK1 and androgen receptor (AR)–negative prostate cancer. (C) DKK1 protein levels in DU145 cell culture medium analyzed with enzyme-linked immunosorbent assay in cells expressing control (sgNT) and DKK1-targeted (sgDKK1) CRISPR guides. (D) and (E) The correlation of [^{18}F]dihydrotestosterone (FDHT) positron emission tomography (PET) uptake serum prostate-specific antigen (PSA) and serum DKK1. (F) Serum levels of DKK1 quantitated in healthy control men and patients with mCRPC. (G) mRNA levels of AXIN2 in the SU2C/PCF cohort of tumor samples. Wnt mutant tumors defined as pathogenic mutations in either APC, CTNNB1, RNF43, ZNRF3, or RSPO2. (H) Comparison of DKK1 mRNA levels by DKK1 amplification status in the SU2C/PCF mCRPC cohort. (I) Comparison of AXIN2 mRNA levels by PSA level and Wnt mutation status in the SU2C/PCF mCRPC cohort. Abi, abiraterone; Enz, enzalutamide; FHCRC, Fred Hutchinson Cancer Research Center; mut, mutated; NS, not significant; RPKM, reads per kilobase per million mapped reads; wt, wild type.

APPENDIX

RNA Sequencing Analysis

Differential expression analysis between high androgen receptor (AR^{high}) and AR^{low} cases was conducted using the limma voom pipeline.[1] Briefly, lowly expressed genes were first removed by only keeping genes that were expressed at ≥ 2 counts per million in ≥ 10 samples. The filtered count matrix was then sent through voom to model the mean/variance trend of the read counts before differential expression analysis. All statistics reported from the RNA sequencing data in this article are the false discovery rate–corrected *P* values (*q* values). Gene set enrichment analysis using gene list preranked by log-fold change was run with 1,000 permutations. Signature of genes upregulated in basal-type prostate cells from Smith et al,[17] neuroendocrine (NE) prostate cancer from Beltran et al,[3] and endothelial-mesenchymal transition from curated hallmark 50-gene sets (Broad Institute: (https://software.broadinstitute.org/gsea/msigdb/index.jsp). CIBERSORT analysis was carried out on a web-based tool (Stanford University: https://cibersort.stanford.edu) and run with 100 permutations using the default LM22 curated set of immune cell gene expression signatures on the file of whole-genome RNA sequencing from the Stand Up to Cancer (SU2C) cohort. RNA sequencing of prostate cancer organoids was carried out as previously described.[14]

Wnt Copy Number Alteration and Mutational Analysis

SU2C cohort. Copy number alteration and mutational data were obtained from 212 patients with available mRNA data from the expanded SU2C/Prostate Cancer Foundation (PCF) cohort.[6] To determine the association between activating mutations of WNT signaling and DKK1 mRNA expression, we first excluded cases with both Dickkopf-1 (DKK1) copy number amplification and mutations of unknown significance in Wnt signaling genes (n = 17). The remaining 195 cases with genomic alterations associated with activation of Wnt signaling were classified as Wnt mutated. These included cases with alterations in APC (oncogenic mutations, homozygous deletions, or biallelic loss), CTNNB1 (oncogenic mutations), RSPO2 (copy number amplifications), and RNF43 and ZNRF3 (homozygous deletions). The remainder were classified as Wnt wild type. To determine whether AR signaling correlated with DKK1 mRNA expression, we further subdivided the cohort into PSA^{low} (lowest quartile of KLK3 mRNA expression) and PSA^{high} (remainder of cases above the 25th percentile of KLK3 mRNA expression).

University of Washington/Fred Hutchinson Cancer Research Center validation cohort. Samples collected through the rapid autopsy program at the University of Washington/Fred Hutchinson Cancer Research Center were as described previously.[4] Samples were determined to be AR negative if their AR target gene, enrichment score (ES) < –0.5, and AR expression were very low or absent; otherwise, they were categorized as AR positive.[4] Samples with NE ES > 0.5 were considered NE positive and otherwise, NE negative. The activity scores and ESs were calculated with R statistical software (R Foundation for Statistical Computing, Vienna, Austria) using the gene set variation analysis package Bioconductor 3.4.[1] The 20-gene AR signature was described previously[2]; the NE signature is a custom set of 10 genes.

DKK1 Methylation Analysis

mCRPC samples from anatomically distinct metastatic sites (at least two per patient) were procured as part of the Johns Hopkins rapid autopsy program and were obtained through the Prostate Cancer Biorepository Network (Prostate Cancer Biorepository Network: https://prostatebiorepository.org).[31,32] Nucleic acids were extracted as described previously.[33] For DKK1 locus-specific DNA methylation analyses, a previously validated assay combining methylated DNA precipitation and methylation-sensitive restriction enzyme digestion (COMPARE-MS) was used.[34] In brief, DNA samples were digested with AluI and HhaI (New England Biolabs, Ipswich, MA), and methylated DNA fragments were enriched using recombinant MBD2-MBD (Clontech, Mountain View, CA) immobilized on magnetic Tylon beads (Clontech). Precipitated DNA containing methylated DNA fragments was eluted and subjected to quantitative real-time polymerase chain reaction (RT-qPCR) using IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and primers specific to a CpG island in the first intron of *DKK1* (forward: 5′-CGGGTACCTGGACGTCTG; reverse: 5′-CTGCAG GCGAGACAGATTT). For quantitative assessment of locus-specific methylation levels, cycle threshold (ct) values of the samples of interest were normalized to ct values of the positive control (in vitro fully methylated male genomic DNA), and calculated methylation indices (range, 0.0-1.0) were used to derive methylation heat maps.

Cell culture. DU145 were grown in DMEM with 10% fetal bovine serum (FBS), L-glutamine. LNCaP and LNCaP-AR were grown in RPMI supplemented with 10% FBS, L-glutamine. LNCaP-abl cells were grown in RPMI supplemented with 10% charcoal stripped FBS, L-glutamine. The PC3 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown in F-12K Media (#30-2004; ATCC) with 10% FBS. MSK prostate cancer organoid cultures were grown as previously described.[14] Retroviral AR overexpression construct (pWZL/AR) was provided by William Hahn, MD, PhD (Dana-Faber Cancer Institute, Boston, MA) and was described previously.[35] DKK1 protein levels in cell culture medium were quantitated using a DKK1 enzyme-linked immunosorbent assay (ELISA) kit (catalog #DKK100; R&D Systems, Minneapolis, MN). Human DKK1, FKBP5, and GAPDH RT-qPCR primers were obtained from QIAGEN (Hilden, Germany; QT00009093, QT00056714, QT00079247). AXIN2 primers were forward: 5′-GAGTGGACTTGTGCCGACTTCA and reverse: 5′-GGTGGCTGGTGCAAAGACATAG. RNA was isolated with the RNAeasy Minikit (QIAGEN) and converted to cDNA with the Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) using 1 μg of RNA. RT-qPCR was carried out using Fast Syber Green Master Mix on a QuantiStudio 6 Flex (Life Technologies, Carlsbad, CA), with data represented as averages relative to RPL19 (Δ#1) and control samples (Δ#2) using the $2^{-\Delta\Delta CT}$ method.

Tumor models. Both SCID (CBySmn.CB17-*Prkdc*^{scid}/J) and NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice (10 per group) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions at SmartLabs (Cambridge, MA). Animal use in this investigation conformed with the guidelines of the Committee on Animals of SmartLabs as well as with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication no. 85-23). Both strains of mice were inoculated subcutaneously with 0.75×10^6 PC3 human prostate cancer cells obtained from ATCC. When tumors reached a volume of 75-100 mm^3 and randomized, intraperitoneal treatment of DKN-01 or IgG4 control was initiated twice a week at 5 mg/kg. For NK depletion in SCID mice, animals were administered 50 μL anti-asialo GM1 antibody (Wako, Richmond, VA) intraperitoneally 3 days before DKN-01 or IgG4 treatment initiation and every 5 days thereafter until study completion. NK depletion was confirmed by flow cytometric evaluation with antibodies to CD49b and NKp46.

DKK1 ELISA. All patients signed consent forms on basis of an institutional review board–approved protocol, blood samples were obtained at the time of newly diagnosed metastatic castrate-sensitive prostate cancer, and a matched sample was collected at the time of progression of mCRPC. DKK-1 was measured using a microfluidic fluoroimmunoassay on the Ella analyzer (ProteinSimple, San Jose, CA) validated in the Memorial Sloan Kettering Clinical Chemistry Laboratory according to standards set by the Clinical Laboratory Improvement Amendments of 1988 and New York State Department of Health. Human investigations were performed after approval by a local human investigations committee and in accordance with an assurance filed with and approved by the Department of Health and Human Services, where appropriate.