Pyruvate kinase L/R links metabolism dysfunction to neuroendocrine differentiation of prostate cancer by ZBTB10 deficiency

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INTRODUCTION

Prostate cancer (PCa) represents a major cause of urologic-related morbidity and mortality in men worldwide [1]. The androgen receptor (AR) plays an important role in the basic mechanisms underlying PCa initiation and progression [2]. Therapeutic interventions with androgen-deprivation therapy (ADT) may lead to the development of metastatic castration-resistant PCa (CRPC) by activating androgen-independent pathways [3]. A subset of tumor cells from CRPC patients receiving ADT or current AR pathway inhibition (ARP1) therapy may progress to an AR-negative phenotype, and this is considered to result from an accumulation of neuroendocrine (NE) and stem cell characteristics [4]. The most important histological variation of NE-like PCa is NE differentiation (NED) of ADT-resistant PCa. We demonstrated that PKLR acts as a modulator to activate NED in PCa enhancement by loss of ZBTB10, thereby enabling PCa cells to mount a glycolysis response essential for therapeutic resistance. Our findings highlight the broad relation between NED and metabolic dysfunction to provide gene expression-based biomarkers for NEPC treatment.

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Neuroendocrine differentiation (NED) frequently occurs in androgen-deprivation therapy (ADT)-resistant prostate cancer (PCa) and is typically associated with metabolic pathway alterations, acquisition of lineage plasticity, and malignancy. There is no conventional therapeutic approach for PCa patients with NED pathologic features because the molecular targets are unknown. Here, we evaluated the regulatory mechanism of NED-associated metabolic reprogramming induced by ADT. We detected that the loss of the androgen-responsive transcription factor, zinc finger, and BTB domain containing 10 (ZBTB10), can activate pyruvate kinase L/R (PKLR) to enhance a NED response that is associated with glucose uptake by PCa cells. PKLR exhibits a tumor-promoting effect in PCa after ADT, but ZBTB10 can compensate for the glucose metabolism and NED capacity of PKLR through the direct transcriptional downregulation of PKLR. Targeting PKLR by drug repurposing with FDA-approved compounds can reduce the aggressiveness and NED of ADT-resistant PCa. We demonstrated that PKLR acts as a modulator to activate NED in PCa enhancement by loss of ZBTB10, thereby enabling PCa cells to mount a glycolysis response essential for therapeutic resistance. Our findings highlight the broad relation between NED and metabolic dysfunction to provide gene expression-based biomarkers for NEPC treatment.

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We sought to understand the metabolic basis of PKLR that influences the NED properties of tumors to provide a foundation for developing effective targeted strategies for PCa.

As the incidence of NEPC has increased following potent ARPI treatment [16], regulatory pathways involved in ARPI-induced activation of glycolytic enzymes urgently need to be investigated. PKLR is one of the key regulators of metabolic reprogramming supporting energy production [10], and it is important that we further understand the molecular basis of PKLR and define its role in the development of NED and therapeutic resistance of PCa. In this study, we investigated the role of PKLR in PCa and established the molecular basis of the tumor-promoting effects of PKLR that are associated with NEPC development after AR-targeted therapy. We provide evidence showing that loss of the androgen-responsive transcription factor, zinc finger, and BTB domain containing 10 (ZBTB10), is associated with PKLR upregulation and the NED of PCa. We demonstrated that overexpression of ZBTB10 can suppress the glycolysis and oncogenic effects of PKLR, indicating the tumor-suppressive role of ZBTB10 in PCa. We also showed that pharmacological inhibition of PKLR decreases tumor growth and NED progression in multiple in vitro and in vivo models of ADT-resistant and NE-like PCa cells, providing a potential approach for treating therapy-resistant PCa.

**RESULTS**

Upregulation of PKLR is involved in NED of PCa after ADT

To determine the role of PKLR in PCa progression, we used a tissue microarray (TMA) obtained from the Department of Pathology at Duke University School of Medicine (Durham, NC, USA), which is composed of normal tissues (n = 16), adenocarcinomas with a Gleason score of ≤7 (n = 81), adenocarcinomas with a Gleason score of ≥8 (n = 19), and SCPC specimens (n = 8) to determine PKLR expression in different grades of PCa. Interestingly, according to immunohistochemical (IHC) staining, we found that high-grade tumors had moderate PKLR expression, and most SCPC cases had high PKLR expression, compared to low-grade tumors and normal prostate tissues (Fig. 1A, B). In studying the clinical relevance of PKLR, we found that prostate tumors from the Taylor PCa dataset [17] with higher PKLR expression were associated with metastasis (Fig. 1C), and a high pathological grade based on the Gleason score (PathGGS) (Fig. 1D). Importantly, levels of PKLR were higher in PCa patients with NEPC compared to patients with adenocarcinomas in Aggarwal datasets [6] (Fig. 1E). Moreover, upregulation of PKLR was positively associated with an NEPC gene signature [18] in The Cancer Genome Atlas (TCGA) PCa database [19] as confirmed by a gene set enrichment analysis (GSEA) (Fig. 1F).

Furthermore, Kaplan–Meier analyses showed that patients with tumors exhibiting higher PKLR expression had shorter overall survival than those with low PKLR expression in the Taylor [17] and TCGA [19] PCa datasets (Fig. 1G and Supplementary Fig. S1A). These results supported the hypothesis that PKLR overexpression is involved in malignant progression and may drive the NED of PCa. To understand expression patterns of PKLR in connection with the NED of PCa, we examined protein expression levels of PKLR, NE markers (ENO2 CHGA, and androgen-responsive markers (MKX3-1 and KLK3) in a panel of PCa cell lines. We found that the AR-negative PC3 [20] and NE-like NCI-H660 [7] and LASCPC01 [21] cell lines expressed higher PKLR and NE markers and lower androgen-responsive markers than did the AR-positive VCaP, LNCaP, and C4-2 prostate adenocarcinoma cell lines (Fig. 1H). It was shown that NE-like PCa cells are involved in lineage plasticity by stimulating stem cell marker expressions [5]. We found that PKLR modification in PCa cells could alter expressions of NE (CHGA, CHGB, ENO2, and SYP) markers that are positively associated with stem cell (NAINO2 and SOX2) marker expressions and negatively associated with androgen-responsive genes (Fig. 1I and Supplementary Fig. S1B–D). Moreover, combined GSEA results from multiple gene signatures related to neurodevelopment showed positive correlations between PKLR expression and neurodevelopment gene signatures in TCGA PCa dataset according to significant false discovery rates (FDRs) (of <0.25) (GO, KEGG, and Reactome; Supplementary Fig. S1E). These results suggested that PKLR expression may be associated with the NED progression of PCa cells.

It was shown that ADT or more effective AR-targeted therapy may lead to a clinically significant increase in NED-characterized PCa [7]. We found that AR-positive LNCaP and C4-2 cells treated with charcoal-stripped serum (CSS)-containing medium to mimic ADT had higher levels of PKLR, which were positively associated with NE and negatively associated with androgen-responsive markers. However, decreased protein levels of NE markers and increased expressions of androgen-negative markers were found in PKLR-KD cells regardless of CSS-containing medium treatment (Fig. 1K).

Moreover, when LNCaP and C4-2 cells were exposed to long-term treatment with MDV3100 (see also LNCaP-MDVR and C4-2-MDVR cells), a higher level of PKLR was associated with increased NE and stem cell markers and decreased androgen-responsive markers; however, PKLR-KD abolished the alterations of these markers (Fig. 1L and Supplementary Fig. S1F). These results suggest that partial NED after ADT driven by PKLR may be associated with increased stem cell markers.

**Loss of ZBTB10 promotes PKLR-driven NED of PCa after ADT**

In order to understand how the suppression of AR signaling upregulates PKLR expression, we analyzed PKLR expression with gene signatures that reflected androgen-responsive genes in the Taylor [17] and TCGA PCa datasets [19]. We found that tissues expressing high levels of PKLR were negatively associated with gene signatures of upregulated androgen responsiveness (Nelson [22], Wang [23], and Hallmark) by a GSEA in both datasets (Supplementary Fig. S2A, B). We focused on AR signaling components that were negatively correlated with PKLR according to GSEA negative rank metric scores in both PCa datasets with PKLR upregulation, and a Venn diagram showed that MAFC, ACCL3, APPBP2, PIAS1, and ZBTB10 genes overlapped among three androgen-responsiveness signatures (Supplementary Fig. S2C, D). Five candidate genes associated with PKLR were validated by a Pearson correlation analysis, and we found that CACL3, APPBP2, PIAS1, and ZBTB10 were significantly negatively correlated with PKLR based on significant p values (of <0.0001) in the two PCa datasets (Supplementary Fig. S2E). To determine the effect of ADT on the expressions of these five genes, we examined the mean expression values of these five genes in messenger (m)RNA expression profile data from LNCaP cells cultured during 11 months of androgen deprivation. Results showed that a decrease in ZBTB10 was significantly associated with an increase in PKLR expression compared to other genes (GDS3358, Fig. 2A and Supplementary Fig. S2F). We also observed increased PKLR and decreased ZBTB10 expressions in post-ADT patients compared to pre-ADT patients from mRNA expression profile data (GSE48403, Fig. 2B). Next, we examined protein levels of ZBTB10 in a panel of prostate cell lines and found that AR-negative PC3 and NE-like LASCPC01 cells expressed lower ZBTB10 than AR-positive VCaP, LNCaP, C4-2, and 22Rv1 adenocarcinoma cells (Fig. 2C). We also found an inverse relationship between mRNA levels of ZBTB10 and PKLR in various PCa cells (Supplementary Fig. S3A). To mimic ADT, we treated AR-positive LNCaP and C4-2 cells with MDV3100 for 1–5 months, and results showed that MDV3100-treated cells exhibited significant induction of PKLR and reduction of ZBTB10 compared to parental cells (Fig. 2D and Supplementary Fig. S3B). We also found that upregulation of PKLR was associated with increases in NE and stem cell markers and decreased expressions of ZBTB10 and androgen-responsive genes following CSS-containing medium (to mimic ADT) treatment, whereas the effects of ADT were abolished in the AR ligand-resistant PCa.
Dihydrotestosterone (DHT)-rescued cells (Fig. 2E, F and Supplementary Fig. S3C). We further examined the effect of ADT on PKLR and ZBTB10 expressions in clinical samples, which consisted of tissue specimens from 17 PCa patients before and after being treated with ADT collected from Taipei Medical University-Wan Fang Hospital (Taipei, Taiwan). The IHC analysis showed higher levels of PKLR in prostate tumors in patients who received ADT compared to the same patients before ADT; however, ZBTB10 levels were higher in patients before ADT and lower in patients after ADT (Fig. 2G, H). The mean expression correlation of ZBTB10 was validated from the Taylor PCa dataset [17], which showed that ZBTB10 was negatively correlated with NE and stem cell markers and positively correlated with AR-responsive gene expressions according to a Pearson coefficient correlation analysis (Supplementary Fig. S3D). Moreover, tissues expressing high levels of ZBTB10 were negatively associated with upregulated neurodevelopment gene signatures and positively associated with upregulated AR signaling responses as analyzed by GSEAs in TCGA PCa dataset according to significant FDRs (Supplementary Fig. S3E). In summary, these results indicated that inhibition of AR signaling may induce downregulation of ZBTB10 which was correlated with upregulation of PKLR in PCa cells.

**Androgen-activated ZBTB10 directly and negatively regulates PKLR expression**

ZBTB10 is a transcriptional inhibitor of regulatory genes of the specific protein (SP) family of transcription factors [24], and was shown to play a tumor-suppressive role in ovarian epithelial
cancer cells [25]. We hypothesized that ZBTB10 may act as a transcriptional repressor of PKLR. We downloaded chromatin immunoprecipitation–ChIP (ChIP)-sequencing data from GEO (GSE105183) and analyzed it with Genome Browser (Genomics Institute, UCSC). Results showed that ZBTB10 may bind to multiple sites of the PKLR gene (Supplementary Fig. S4A). To examine the role of ZBTB10 in mediating expressions of PKLR-associated NE markers, ZBTB10 CDNA was stably transfected into AR-negative PC3 and NE-like LSPC01 cells and results showed that protein and mRNA levels of PKLR and NE markers were significantly reduced (Fig. 3A–C). Conversely, AR-positive cells with ZBTB10-KD exhibited significantly increased PKLR and NE markers and decreased expressions of AR-responsive markers (Supplementary Fig. S4B–D). We searched for sequences resembling the ZBTB10 response element (ZRE) [26] in PKLR regulatory sequences. We found four putative ZREs relative to the transcriptional start site of PKLR (Fig. 3F). A ChIP assay was performed and we found that ZRE2 and ZRE3 had significantly decreased ZBTB10-binding ability on PKLR after ADT of cells (Fig. 3E). In addition, ZBTB10 overexpression in PC3 cells increased the binding ability of ZBTB10 to ZEB2 and ZEB3 (Fig. 3F, left); however, ZBTB10-KD in LNCP cells reduced the binding ability of ZBTB10 to ZEB2 and ZEB3 (Fig. 3F, right). Reporter assays were performed and we found that CSS-containing medium-treated C4-2 and LNCaP cells showed significantly increased reporter gene activity relative to untreated cells, whereas cells with additional DHT treatment showed decreased reporter gene activity (Fig. 3G). Moreover, ZBTB10 overexpression downregulated reporter activity, while ZBTB10-KD upregulated reporter activity (Fig. 3H), supporting ZBTB10 transcriptional downregulation of PKLR. We further used a PKLR regulatory sequence-GFP reporter construct in which putative ZRE sites were individually or doubly mutated (Fig. 3D). Results showed that a significant increase in reporter activity was detected at the reporter harboring ZRE2 and ZRE3 mutations (ZRE2M, ZRE3M, and ZRE23M) compared to the ZRE-WT reporter in PC3 cells (Fig. 3I). Moreover, ZBTB10 overexpression downregulated ZRE-WT reporter activity in PC3 cells, while the ZRE2M, ZRE3M, and ZRE23M mutants reduced the suppressive effect of ZBTB10 compared to ZRE1M or ZRE4M reporters, especially the double-mutant ZRE23M (Fig. 3I). These results indicated that ZBTB10 may directly interact with the promoter region of PKLR, and downregulation of ZBTB10 through ADT may induce the accumulation of PKLR.

Loss of ZBTB10 induces PKLR-mediated glucose metabolism and NED in PCa

Previous studies showed that dysregulation of AR signaling can promote glucose metabolism in PCa [27]; however, the mechanism responsible for the abnormalities in glycolytic enzymes caused by ADT-induced metabolic gene and metabolic reprogramming in regulating NEPC development is still unclear. We found that AR-positive LNCaP and C4-2 cells treated ADT showed increased glucose consumption and also higher lactate secretion and pyruvate levels compared to control cells (Fig. 4A–C). Upregulation of PKLR was confirmed in cells treated with CSS-containing medium, but not in CSS-treated PKLR-KD cells (Fig. 4D). Moreover, AR-negative PC3 and NE-like LSPC01 cells expressing PKLR-KD showed decreased glucose consumption, lactate secretion, and pyruvate levels relative to control cells (Supplementary Fig. S5B). We next analyzed PKLR levels in ZBTB10-overexpressing LNCaP and C4-2 cells in response to ADT. Although ADT increased endogenous PKLR expression, cells overexpressing ZBTB10 exhibited a decrease in PKLR regardless of ADT treatment (Fig. 4E). We also found that ADT could induce higher glucose consumption and lactate and pyruvate levels, while ZBTB10 overexpression reduced these effects (Fig. 4F–H). We further analyzed the extracellular acidification rate (ECAR) in PKLR-KD cells relative to ADT using a Seahorse XFe24 analyzer. Results showed that ECAR values significantly increased in cells responsive to ADT (NC/CSS vs. NC/CSS), while lower ECAR values were found in C4-2 cells with PKLR-KD compared to control cells in the presence or absence of ADT (NC/CSS vs. shPKLR/CSS or NC/CSS vs. shPKLR/CSS) (Fig. 4I). Interestingly, PC3 and LSPC01 cells expressing PKLR-KD had lower ECAR values (Supplementary Fig. S5C, D), supporting the role of PKLR in activating glucose metabolism in AR-negative and NE-like PCa cells. Consistently, we found that cells overexpressing ZBTB10 exhibited reduced ECAR values regardless of ADT conditions (EV vs. ZBTB10/CSS or EV + CSS vs. ZBTB10/CSS) (Fig. 4I). We further rescued PKLR expression into a ZBTB10 cDNA vector (a ZBTB10 cDNA vector without the ZBTB10 regulatory sequence)-expressing LNCaP and C4-2 cells to evaluate the role of PKLR. Results showed that cells overexpressing ZBTB10 exhibited reduced expressions of endogenous PKLR, while rescued PKLR did not alter the protein levels of exogenous ZBTB10 (Fig. 4K). We also found that overexpressing ZBTB10 can reduce mRNA levels of PKLR, and NE and stem cell markers and upregulation of androgen-responsive genes, whereas rescue of PKLR in ZBTB10-expressing cells was associated with upregulation of NE and stem cell markers and reduction of an androgen-responsive gene (Fig. 4L and 4M).
Supplementary Fig. S6A). Moreover, glucose consumption and lactate and pyruvate levels were found to be reduced in ZBTB10-overexpressing cells compared to control cells, and rescue of PKLR abolished the effects of ZBTB10 (Supplementary Fig. S6B). We also found that cells with ZBTB10 overexpression had significantly lower ECAR values compared to control cells, whereas upregulation of ECAR values was found in PKLR-recused cells regardless of ZBTB10 overexpression (Fig. 4M and Supplementary Fig. S6C). In addition, we found that cells overexpressing ZBTB10 had lower OCR values, whereas ZBTB10-expressing cells rescued with PKLR had higher OCR values (Fig. 4N and Supplementary Fig. S6D), suggesting a role of PKLR involved in activating oxidative phosphorylation (OXPHOS) in PCa cells. In summary, these data suggested that PKLR may compensate for the ZBTB10-suppressed NED and glucose metabolism of PCa.

**PKLR compensates for the tumor-suppressive effect of ZBTB10**

Regarding the functional role of PKLR in PCa progression, we found that C4-2 cells overexpressing PKLR exhibited statistically increased growth rates in vitro (Fig. 5A). However, PC3 cells with PKLR-KD exhibited a reduced growth rate compared to cells that carried the control vector (Fig. 5B). Moreover, a three-dimensional...
sphere-formation assay showed that sphere formation in Matrigel was significantly higher or lower in C4-2 and PC3 cells with PKLR overexpression or PKLR-KD, respectively (Fig. 5C, D). Immunoblotting confirmed the expression of the PKLR protein in cells with PKLR modification (Fig. 5E). Furthermore, we found that ADT-resistant LNCaP cells had higher cell proliferation rates compared to parental cells, whereas cells with PKLR-KD or ZBTB10 overexpression had lower cell proliferation rates regardless of ADT resistance (Supplementary Fig. S7A, B). In addition, we also found that ADT-resistant C4-2 cells had induced numbers of spheres, whereas both PKLR-KD and ZBTB10 overexpression reduced numbers of spheres in parental and ADT-resistant cells (Supplementary Fig. S7C, D). To evaluate the potential tumor-suppressive effect of ZBTB10 in downregulating PKLR-driven PCa progression, we used a stable ZBTB10-expressing clone and compared it to ZBTB10-expressing cells rescued with the PKLR cDNA vector in AR-positive LNCaP and C4-2 cells. An MTT analysis revealed that ZBTB10-overexpressing cells showed a significantly lower growth rate compared to cells carrying the control vector; however, the cell proliferation rate was higher in PKLR-rescued ZBTB10-expressing cells (Fig. 5F, G). Consistently, we found that cells with ZBTB10 expression had decreased sphere formation, while ZBTB10-expressing cells rescued with a PKLR cDNA vector exhibited an increased effect (Fig. 5H). We next monitored cell
migration and invasion of ZBTB10-expressing cells compared to PKLR-rescued ZBTB10-expressing cells, and found that cells expressing ZBTB10 showed reduced cell migration and invasion; however, ZBTB10-expressing cells rescued with PKLR exhibited significantly higher cell migration and invasion (Fig. 5I, J). Decreased levels of proliferation markers (MKI67 and PCNA) and epithelial-mesenchymal transition (EMT) markers (SNAI1, TWIST1, and VIM) were found in ZBTB10-overexpressing cells; whereas
higher levels of these markers were found in PKLR-rescued cells regardless of ZBTB10 expression (Fig. 5K, L). These data indicated that ZBTB10 may be a tumor-repressive factor that decreases the malignancy and progression of PCa cells; however, PKLR can compensate for the tumor-suppressive role of ZBTB10.

Targeting PKLR suppresses tumor growth and NED of PCa
Since there are no PKLR inhibitors approved for clinical use, we conducted in-house drug testing and analysis to study the promising therapeutic inhibitory effects of approved drugs that target PKLR. In order to find PKLR inhibitors and study the binding mode of PKLR with approved drugs, a molecular docking analysis was performed. Molecular docking was carried out with iGemDock V2.1 [28], using the small-molecule modules downloaded from the ZINC15 database [29], and structural information of the docked PKLR protein (active site, binding site, and allosteric activating site) was obtained from UniProt. When docking was completed, the highest docking simulation of each compound was selected and ranked by the calculated free energy. Putative PKLR inhibitors were further screened based on their cytotoxicity to PCa cells and normal prostate epithelial cells, followed by their ability to inhibit cell growth and sphere formation in vitro. Interestingly, we found that higher doses of vilanterol and saquinavir significantly reduced the viability of AR-negative PC3 and NE-like LASCPC01 cells compared to other drugs, while vilanterol and saquinavir did not significantly decrease the viability of the normal PZ-HPV-7
prostate epithelial cell line or AR-positive LNCaP, C4-2, and 22Rv1 cells according to MTT assays (Fig. 6A and Supplementary Fig. S8A). To test the specificity of targeting PKLR, C4-2 cells with PKLR overexpression (C4-2/PKLR) or MDV3100-resistance (C4-2-MDVR) were treated with these small-molecule drugs, and results showed that C4-2/PKLR or C4-2-MDVR cells treated with vilanterol and saquinavir exhibited significantly reduced growth rates compared to cells treated with other drugs (Fig. 6B, C and Supplementary Fig. S8B–D). Vilanterol and saquinavir did not significantly decrease sphere formation of parental C4-2 and...
LNCaP cells (Supplementary Fig. S9A). However, C4-2/PKLR, C4-2-MDVR, PC3, and LASCPC01 cells treated with vilanterol or saquinavir treatment showed reduced sphere formation, compared to cells with DMSO treatment (Fig. 6D). Despite PKLR or ADT inducing glucose metabolism in parental C4-2 and LNCaP cells, both drugs significantly reduced glucose uptake in PKLR-overexpressing or MDV3100-resistant cells; however, neither drug appeared to alter glucose uptake in control cells (Supplementary Fig. S9B, C). Furthermore, C4-2/PKLR, C4-2-MDVR, PC3, and LASCPC01 cells treated with vilanterol and saquinavir exhibited reductions in NE and stem cell markers, but not PKLR (Fig. 6E and Supplementary Fig. S9D), suggesting that vilanterol and saquinavir may be candidate inhibitors of PKLR through directly abolishing functional sites of PKLR. After selection, we identified two candidate PKLR inhibitors, vilanterol and saquinavir, which were further tested in vivo. Mice were subcutaneously injected with LASCPC01 and C4-2-MDVR cells and were further treated with vilanterol or saquinavir after tumor formation (Fig. 6F). We found that mice harboring LASCPC01 or C4-2-MDVR tumor cells with vilanterol or saquinavir treatment exhibited dramatic decreases in tumor growth (Fig. 6G, H and Supplementary Fig. S10A) and lower tumor weights (Fig. 6H, I and Supplementary Fig. S10B), compared to control mice. Interestingly, although we did not see a decrease in PKLR in mice treated with vilanterol or saquinavir, decreased NE (ENO2) and proliferation (MKI67 and PCNA) markers were found in mice after vilanterol or saquinavir treatment, as confirmed by IHC staining of subcutaneous tumors (Fig. 6J and Supplementary Fig. S10C), suggesting that PKLR-targeted therapy might suppress a variety of NED properties as well as growth rates of PCa cells.

**High-grade PCa in patients with reduced ZBTB10 and increased PKLR abundances**

To study relationships between ZBTB10 and PKLR in clinical tissue samples, we analyzed a PCA TMA (Super Bio Chips #CA4) comprising 49 cases of primary PCa. IHC results showed a reverse correlation between ZBTB10 and PKLR in consecutive tissue sections of prostate tumor samples (Fig. 7A–C). Importantly, high-grade tumors (with Gleason scores of ≥8) had greater abundances of PKLR and lower abundances of nuclear ZBTB10, whereas the reverse was seen in low-grade tumors (with Gleason scores of ≤6) (Fig. 7D, E). Furthermore, since high abundances of PKLR were detected in SCPC samples, we found that the same SCPC samples had lower abundances of ZBTB10 compared to PKLR (Supplementary Fig. S11). These results supported our hypothesis that PKLR abundance is positively correlated with loss of ZBTB10 and is associated with a poor prognosis. In summary, our results demonstrated a regulatory mechanism that inhibition of AR signaling in hormone-sensitive PCa through ADT may cause a reduction in ZBTB10, leading to abundances of PKLR-promoting oncogenic signaling pathway components in a subset of patients with hormone-refractory PCa associated with enhanced glucose metabolism and NE characteristics. Targeting PKLR by drug repurposing with small-molecule PKLR inhibitors may reduce the PKLR-driven glycolysis and NED of ADT-resistant PCa (Fig. 7F).

**DISCUSSION**

During the malignant transformation of PCa, the glucose metabolism pathway is frequently abnormal due to the upregulation of genes encoding glycolytic enzymes and pyruvate kinase [8]. PKLR is an isoenzyme of pyruvate kinase encoded by the *PKLR* gene, which is mainly activated in cells during glucose metabolism [10]. We identified a novel signaling pathway that regulates PKLR and provided a specific therapeutic strategy to target ADT-resistant and NE-like PCa cells by pharmacological inhibition of PKLR. We studied key functions of PKLR in regulating glucose metabolism and NED progression in prostate tumors, by illuminating novel functions of PKLR in regulating NED-associated gene expressions. However, a limitation of this study was determining how PKLR regulates gene expression. Our IHC staining results showed that the intensity of nuclear PKLR was increased in high-grade PCa and in PCa samples after ADT resistance, especially a dramatic abundance of nuclear PKLR was seen in aggressive SCPC samples (Figs. 1A, 2G). We hypothesized that PKLR may be translocated into nuclei and act as a transcription factor to enhance its target gene expressions in addition to conducting its kinase function. It was shown that another pyruvate kinase, PKM2, can be translocated into nuclei through activation of the epidermal growth factor receptor (EGFR) pathway [30]. In addition, nuclear PKM2 may also act as a transcription co-activator with hypoxia-inducible factor (HIF)-1α [31, 32]. PKLR might also act similarly to PKM2 and be associated with the hypoxia-HIF-1α pathway in PCa. We found significant alterations in expressions of the AR downstream targets, KLK3 and NKX3-1, in PKLR-modified cells (Fig. 1I and Supplementary Fig. S1B–D). These results suggested that modification of KLK3 and NKX3-1 may be due to direct or indirect regulation of nuclear PKLR. We are currently further investigating the mechanism by which PKLR is involved in nuclear translocation during PCa progression.

Our study aimed to illustrate the oncological role of PKLR in PCa progression and its mechanism of upregulation after ADT. ZBTB10 is a novel member of the ZBTB transcription factors, and its biological role has not been fully elucidated. ZBTB10 serves as a transcriptional inhibitor of SP1 transcription factors and participates in inhibiting tumor activities, including tumor growth, chemoresistance, and migration/invasion [33, 34], suggesting that ZBTB10 should be considered a tumor suppressor. In a genome-wide association study focused on gene loci, ZBTB10 was found to be associated with the sex hormone-binding globulin content [35], which indicates that ZBTB10 might participate in the development of the prostate gland. Through analysis of PCa mRNA expression databases and subsequent in vitro and in vivo experiments, we demonstrated a novel role for ZBTB10, which may be an upstream regulator of PKLR in PCa. Reducing the activity of AR signaling by ADT or pharmacological AR antagonists suppresses the AR, which may downregulate ZBTB10, facilitating PKLR upregulation. This AR/ZBTB10/PKLR axis provides one explanation for the common progression to hormone-refractory PCa after ADT in patients. Our results indicated that loss of ZBTB10 in PCa may be able to support glucose metabolism and NED progression by altering the expression and oncopgenic role of PKLR.

We further assessed the potential of targeted PKLR therapy in aggressive PCa treatment. We screened potential PKLR inhibitors from approved drugs and found two drugs, vilanterol and saquinavir, which could be cytotoxic antagonist targets of NE-like and ADT-resistant cells. When administered to mice bearing LASCPC01 cells, vilanterol was found to effectively inhibit tumor growth, suggesting that vilanterol may be a promising drug for treating NE-characterized PCa. Our study is the first report of vilanterol being used for cancer therapy. Saquinavir is a human immunodeficient virus protease inhibitor (HIV-PI) that inhibits the cleavage of polyproteins synthesized from retroviral genomes [37]. Many studies showed that HIV-PIs are also antitumor agents for lung, breast, colon, and prostate tumors [37]. HIV-PIs are used to block internal proteasome activity, causing ER stress or growth factor signal interference, leading to apoptosis of PCa cells [38]. Notably, saquinavir exhibited growth-inhibitory activity against tumor formation of AR-negative PC3 and DU145 cells [39], suggesting the anti-PCa activity of saquinavir. Our cell viability
results showed that 10, 25, or 50 μM of vilanterol significantly decreased of the viability of PC3 and LASCPC01 cells by more than 50% compared to the normal PZ-HPV-7 prostate epithelial cell line, whereas 10 or 25 μM of saquinavir displayed no significant difference between PC3/LASCPC01 and normal prostate epithelial cells (Fig. 6A). These results indicated a better cytostatic response of vilanterol compared to saquinavir in PCa.

CONCLUSIONS
Our results showed that an inverse relationship exists between AR/ZBTB10 signaling and PKLR-driven glucose metabolism and NED signaling pathways. Under androgen regulation, ZBTB10 is a critical transcriptional factor for the direct repression of regulatory sequences of PKLR, while loss of androgen signaling or inhibition of the AR causes an enhanced malignant...
Fig. 7 ZBTB10 is negatively correlated with PKLR expression. A, B ICC staining of PKLR and ZBTB10 on consecutive sections of a PCa TMA (CA4) in two selected cases. Scale bars, 100 μm. C Correlation analysis of the intensities of PKLR and ZBTB10 of the PCa TMA (n = 49). PKLR expression was negatively associated with ZBTB10-expressing PCa samples. ρ, correlation coefficient; p, two-tailed p value. Significance was determined by correlation XY analyses in GraphPad Prism. D, E Tumor grade association analysis using a Chi-squared test of the CA9 PCa TMA. Intensities of PKLR (D) and ZBTB10 (E) staining were semiquantitatively scored using the H-index as follows: negative, weakly positive, moderately positive, and strongly positive. The p values were calculated by a Chi-squared test performed using SPSS statistical 18.0 software. p < 0.001. F Proposed model for ADT-induced PKLR drives hormone-refractory PCa. Hormone-sensitive PCa activates AR signaling by increasing ZBTB10 leading to increased binding to the PKLR regulatory sequence and mediation of its transcriptional suppression. ADT induced inactivation of the AR-ZBTB10 pathway, leading to an abundance of PKLR through ZBTB10 loss of function. Overexpression of PKLR may upregulate glucose metabolism and NED progression of PCa cells. Targeting PKLR by potential PKLR inhibitors may reduce the PKLR-driven glycolysis and NED of ADT-resistant PCa.

phenotype possibly through inactivation of ZBTB10 and activation of PKLR.

MATERIALS AND METHODS

Cell culture and reagents

Cell lines used in this study were obtained from American Type Culture Collection (ATCC). The culture medium was distinct for each cell line. LNCaP cells were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 (ThermoFisher, 11875-085), with 5% fetal bovine serum (FBS, US origin, EMD Millipore, TMS-013-BKR). C4-2 and PC3 cells were cultured in RPMI-1640 with 5% heat-inactivated FBS. VCaP cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Corning, 10-013-CV) with 5% FBS. LASPCP01 cells were cultured in HITES medium which is composed of RPMI-1640, 10 nM hydrocortisone (Sigma-Aldrich, H8988), 1× insulin/ transferrin/serine (ThermoFisher, 41400-045), 200 nM β-estradiol (Sigma- Aldrich, E2758), and 5% FBS. NCI-H660 cells were cultured in RPMI-1640 medium supplemented with 1× insulin/transferrin/serine (ThermoFisher, 41400-045), 10 nM hydrocortisone (Sigma-Aldrich), 10 nM β-estradiol (Sigma-Aldrich, E2758), 4 mM L-glutamine (Invitrogen), and 5% FBS. The PZ-HPV-7 normal prostate epithelial cell line was cultured in keratinocyte serum-free medium (K-SFM; ThermoFisher, 17005-042) supplemented with 0.05 mg/ml bovine pituitary extract (BPE, ThermoFisher) and 5 ng/ml human recombinant epidermal growth factor (EGF, ThermoFisher). MDV3100/enzalutamide-resistant LNCaP-MDVR and C4-2-MDVR cells were respectively derived from LNCaP and C4-2 cells cultured for 12 months in RPMI-1640 medium with 5% FBS containing 20 μM MDV3100 (Selleckchem, S1250) under standard culture conditions. To mimic ADT, cells were cultured in RPMI-1640 medium with 5% CSS (ThermoFisher, 12676-029)-containing medium for 48 h or treated with 10 μM MDV3100 (Selleckchem, S1250) under standard culture conditions for 48 h. The AR ligand was treated with 10 nM DHT (Sigma-Aldrich) for 24 h. The candidate PKLR inhibitors (vilanterol, saquinavir, fosinopril, and salmeterol) were purchased from MedChemExpress (HY-14300, HY-17005, HY-S00382, and HY-14302), and the concentrations of each candidate drug for cell viability were treated with 0, 1, 5, 10, 25, and 50 μM for 24 h.

Tissue samples

PCa TMA sections from Duke University School of Medicine (Durham, NC, USA) included 16 normal prostatic epithelial samples, 81 adenocarcinomas with a Gleason score of ≤7, 19 adenocarcinomas with a Gleason score of ≥8, and eight SCPC samples, and their use was approved by the Duke University School of Medicine Institutional Review Board (protocol ID: Pro00070193). PCa TMA sections comprising PKLR and ZBTB10 in 49 cases of primary prostate adenocarcinoma were purchased from Super Bio (CA4). Tumor samples of 17 PCa patients before and after ADT treatment were collected from Taipei Medical University-associated Wang Fang Hospital following a protocol based on the Declaration of Helsinki, and the study protocol was approved by the Taipei Medical University Joint Institutional Review Board (approval ID: N202001020).

IHC staining

For IHC staining, slices were deparaffinized and rehydrated, the antigen was retrieved using heat, and slices were stained with the first antibody (PKLR, 1:100, Abcam, ab125697; ZBTB10, 1:200, Invitrogen, PA5-54448; enolase 2 (ENO2), 1:100, Abcam, ab218388; a marker of proliferation Ki-67 (MKI67), 1:200, Abcam, ab15580; and proliferating cell nuclear antigen (PCNA), 1:200, Abcam, ab29) at 4 °C. Then, slices were stained with the second antibody (horseradish peroxidase (HRP) anti-rabbit or HRP anti-mouse, Dako, E0432 or E0433) after proper washing with Tris-buffered saline (TBS) buffer with 0.1% Triton X-100, conjugated with avidin, and colorized with the 3,3′-diaminobenzidine (DAB, Dako, K3468) reagent. Then, slices were dehydrated and mounted with glycerol, and a snapshot was taken with a phase-contrast microscope (Olympus IX73). The intensity of the target gene in a slice was diagnosed by a pathologist, and the intensity was defined as 0 (negative), 1+ (weakly positive), 2+ (moderately positive), or 3+ (strongly positive) for H-index calculation according to the following formula [40]:

\[ H \text{ index} = [1 \times (\% \text{ of cells} \times 1++) + 2 \times (\% \text{ of cells} \times 2++) + 3 \times (\% \text{ of cells} \times 3++)]. \]

Proliferation assay

Cell proliferation was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Santa Cruz, sc-359848), in which 5 x 10^5 cells per well were seeded in 96-well plates and cultured overnight until the next day (day 0), one plate was picked to measure cell numbers, and the remaining ones were continued culturing under standard culture conditions. MTT solution (1 mg/mL at 100 μL/well) was added to the test plates (to a final concentration of 500 μg/mL) and incubated an additional 4 h under standard culture conditions. Later, the MTT-containing medium was replaced with 50 μL/well dimethyl sulfoxide (DMSO) and incubated for 10 min in the dark to completely dissolve the formazan crystals. The optical density at 570 nm (OD570) was measured in each well with a microplate reader (BioTek). Relative cell numbers are presented as the OD570 ratio normalized to day 0.

Three-dimensional sphere-formation assay

A protocol of three-dimensional growth in Matrigel was followed from our previous study with slight modification [41]. In brief, 500 cells/well of desired cells were suspended in 50 μL of complete medium followed by mixing with an aliquot of standard Matrigel matrix (Corning, 354234). Then, the mixture was loaded into the bottom edge of a 24-well plate and incubated overnight for aggregation. The next day (day 0), 2 mL of culture medium was loaded into the wells and cultured for 1 week. To evaluate the cytotoxicity of candidate PKLR inhibitors, LNCaP, C4-2, C4-2/PKLR, C4-2-MDVR, PC3, and LASPCP01 cells were treated with 10 μM of vilanterol or saquinavir for 1 week. Finally, tumorspheres in each well were observed, a snapshot was taken with phase-contrast microscopy (Olympus), and the spheres were counted.

Tumorigenicity assays in mice

The protocol of the in vivo tumorigenicity assay was based on Guidelines for Care and Use of Laboratory Animals from the Council of Agriculture, Executive Yuan, Taiwan and was approved by the Taipei Medical University Institutional Animal Care and Use Committee (approval ID: LAC-2020-0032). Six-week-old CnNcXgC-x/ICN1 male mice were purchased from the National Laboratory Animal Center (NLAC, Taipei, Taiwan). Mice were randomized into three groups and subcutaneously injected with 10^6 cells/site (suspended in 100 μL of an aliquot mixture of Matrigel matrix and culture medium) of LASPCP01 and C4-2-MDVR cells in the right side of the flank, and the tumor diameter and mouse weight were monitored once a week. One month after injecting the cells, the mice were intraperitoneally injected with DMSO, vilanterol (25 mg/kg), or saquinavir (25 mg/kg) twice a week for 4 weeks under double-blind conditions. The tumor diameter was converted into a volume according to the following formula [43]:

\[ V = 0.1256 \times W \times H \times L, \]

where V, H, W, and L are the volume, height, width, and length, respectively.

ChiP assay

A ChiP assay was carried out using the EZ-Magna ChiP™ IP kit A (Sigma- Aldrich, 17-10086) following the protocol recommended in the manual.
After treatment, 10^6 cells were fixed with 1% paraformaldehyde/complete medium for 10 min followed by termination of fixing by incubating with 125 mM glycine buffer for 5 min. Fixed cells were washed with PBS with proteinase and phosphatase inhibitors in a freezer. Then, cells were scraped under PBS buffer, and the debris was collected. Chromatin within the debris was released using the lysis buffer in the kit and was disrupted into 150 mg sonication (Sonica). Chromatin–protein complexes were labeled with 10 ng of an anti-ZBTB10 antibody (Abcam, ab117786), anti-acetyl-histone H3 antibody (positive control, Novus, NB300–221), or normal rabbit immunoglobulin G (IgG) (negative control, Santa Cruz, sc–2027) followed by enrichment using protein A–coated magnetic beads. Chromatin was released from the complexes by proteinase K (Sigma–Aldrich, 124568) following heat inactivation, and were identified by a reverse-transcription quantitative polymerase chain reaction (RT-qPCR). ChIP antibodies and qPCR primers are listed in Supplementary Table 54. For the ChIP-sequencing analysis, ChIP-sequencing data were downloaded from Gene Expression Omnibus (GEO) (GSE105183) and analyzed by Genome Browser (Genomics Institute, UCSC, CA, USA).

**Promoter reporter assay**

ZBTB10 response elements (ZREs) are located upstream of human PKLR on chromosome 17 (155297844 (ZRE3: –1998), and 155299967 (ZRE4: +125) at GRCh38. These regulatory sequences with response element green fluorescence protein (GFP) reporter vectors were constructed using the pGreenFire1:ISRE Lentivector (System Biosciences, TR016PA–P). Cells (5 × 10^5 cells/well) in 12-well plates were transiently transfected with 1 µg of the wild-type (WT)- and desired cell lines (at about 90% confluence) at 6 h after the sequential addition of inhibitors of mitochondrial function: 1 µM oligomycin, 0.75 µM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), and a combination of rotenone and antimycin A (at 0.5 µM each).

**Migration and invasion assays**

Migration and invasion assays followed protocols in the literature with slight modifications [43]. For migration, totals of 3 × 10^5 cells/well were suspended in serum-free medium and inoculated in 24-well Boyden chamber (8-µm pore size), and the chamber was put into a 24-well culture plate. For the invasion assay, totals of 2 × 10^5 cells/well were inoculated in the same Boyden chamber pre-coated with 200 µg/ml Matrigel matrix (Corning, 354234). Matrigel-coated transwell dishes were prepared by adding 200 µl of C5s-containing medium diluted with Matrigel. The lower chamber was filled with 600 µl of the complete medium outside the chamber. The plates were incubated for 24 h for migration and 8 h for an invasion before staining the chamber with a 0.5% crystal violet/methanol/ PBS solution for 20 min. After washing with PBS, cells in the chamber which had not invaded were removed with cotton swab, and invaded cells on the underside of the membrane were counted and quantified in five medium-power fields for triplicate replicates.

**Data Availability**

The reagents used in the current study are available from the corresponding author on reasonable request.

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