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Down-regulation of NF-κB signals is involved in loss of 1α,25-dihydroxyvitamin D_3 responsiveness

Bo-Ying Bao[a,b,c,1], Huei-Ju Ting[a,1], Jong-Wei Hsu[a], Sayeda Yasmin-Karim[b], Edward Messing[a], Yi-Fen Lee[a,*]

[a] Department of Urology and Pathology & Laboratory Medicine, Rochester, NY 14642, USA
[b] Department of Chemical Engineering, University of Rochester Medical Center, Rochester, NY 14642, USA
[c] School of Pharmacy, China Medical University, Taichung 404, Taiwan

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ABSTRACT

Vitamin D anti-tumor effect is often found reduced in the late stages of cancer. To uncover vitamin D resistance mechanism, we established a vitamin D-resistant human prostate cancer LNCaP cell line, LNCaP-R, by chronic exposure of cells to 1α,25-dihydroxyvitamin D_3 (1,25-VD). The vitamin D receptor (VDR)-mediated transcriptional activity was reduced in LNCaP-R, whereas VDR expression level and DNA-binding capacity were similar compared to parental cells (LNCaP-P). The expressions of the key factors involved in VDR transactivity, including CYP24A1 and VDR-associated proteins are all increased in LNCaP-R cells, and yet treatment with ketoconazole, P450 enzymes inhibitor, as well as trichostatin A (TSA), a histone deacetylase inhibitor, did not sensitize LNCaP-R cells response to vitamin D, suggesting that neither a local 1,25-VD availability, nor VDR-associated proteins are responsible for the vitamin D resistance. Interestingly, nuclear factor-kappaB (NF-κB) signaling, which is critical for 1,25-VD/VDR activity was found reduced in LNCaP-R cells, thereby treatment with NF-κB activator, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), can sensitize LNCaP-R vitamin D response. Together, we conclude that NF-κB signaling is critical for vitamin D sensitivity, and dysregulation of this pathway would result in vitamin D resistance and disease progression.

1. Introduction

Prostate cancer (PCa) is one of the most common types of cancer among American men. Despite the progress made in understanding the biology of this disease, the management of hormone refractory prostate cancer (HRPC) remains a significant clinical challenge. The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25-VD), has been shown to modulate growth, differentiation, and apoptosis of a variety of normal and transformed cells, such as breast, colon, myeloid, and prostate [1–4]. In clinical trials, 1,25-VD has been used as an adjuvant therapy for human PCa in combination with docetaxel, and has shown promising results in PSA response, time to progression, and survival in HRPC patients [5]. However, 1,25-VD responsiveness is frequently found to be lost during the late stages of cancer progression [6–9], and the detailed mechanisms remain to be determined.

Vitamin D resistance had been shown in many cell types, and numerous mechanisms have been found. 1,25-VD can be metabolized by 1,25-VD 24-hydroxylase (CYP24A1) to a less active form, and increased expression of CYP24A1 has been linked to reduced 1,25-VD sensitivity [10]. Because the major genomic actions of 1,25-VD are mediated by the vitamin D receptor (VDR), alterations on its sequence [11–13], expression [14,15], phosphorylation [16], and nuclear localization [16,17], have all been implied to be involved in vitamin D resistance. The 1,25-VD-bound VDR recognizes vitamin D response elements (VDREs) by forming a heterodimeric complex with the retinoid X receptor (RXR) in the promoters of vitamin D-responsive genes and regulates transcription. In rat osteosarcoma cells, accelerated and aberrant RXR degradation could cause resistance to the antiproliferative effects of 1,25-VD [18]. The classical hereditary vitamin D-resistant rickets patients who have normal VDR expression and function, might be caused by a constitutive overexpression of heterogeneous nuclear ribonucleoprotein (hnRNP) that competed

Abbreviations: 1,25-VD, 1α,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; VDRE, vitamin D response element; PCa, prostate cancer; SRC-1, steroid receptor coactivator-1; DRIP-205, VDR-interacting protein-205; NCoR1, nuclear receptor corepressor 1; SMRT, silencing mediator for retinoid and thyroid hormone receptors; TSA, trichostatin A; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; NF-κB, nuclear factor-kappaB; MAPK, mitogen-activated protein kinase; AP-1, activator protein-1.

* Corresponding author at: Department of Urology, University of Rochester Medical Center, 601 Elmwood Avenue, Box 656, Rochester, NY 14642, USA.
Tel.: +1 585 275 9702; fax: +1 585 756 4133.
E-mail address: yifen_lee@urmc.rochester.edu (Y.-F. Lee).
[1] These authors contributed equally to this work.

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with VDR/RXR binding to the VDRE [19,20]. Many coregulators have been identified to modulate the transcriptional activity of VDR. Coactivators, such as steroid receptor coactivator-1 (SRC-1) and VDR-interacting protein-205 (DRIP-205), contain histone acetylase activity that help chromatin remodeling and facilitate transcription. Corepressors, such as nuclear receptor corepressor 1 (NCoR1), silencing mediator for retinoid and thyroid hormone receptors (SMRT), ALIEN, and ligand dependent nuclear receptor corepressor (LCOR), recruit a complex with histone deacetylase (HDAC) activity and repress transcription of target genes. Our previous study showed that increased expression of the corepressors, NCoR1 and SMRT, result in reduced antiproliferative response to vitamin D [8]. In addition, a proteomics screening identified 14 differentially expressed proteins, which might dictate vitamin D resistance in breast cancer cells, such as down-regulation of caspases (caspase-7 and -14), and up-regulation of mitogenic signals [phospho-p38 and Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 2 (MEK2)] [21]. However, additional work is needed to determine their relevance to 1,25-VD resistance.

To understand the molecular mechanisms that cause vitamin D resistance, there have been several studies in the breast cancer field using vitamin D-resistant human breast cancer MCF-7 cell lines, MCF-7^{D3Res} and MCF-$7/VD^{R}$, as models [22–24]. Although lower VDR transcriptional activity and treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) could sensitize vitamin D-resistant MCF-7 variants to the effects of 1,25-VD [22–24], the detailed mechanisms underlying these effects remain unclear. In an attempt to gain more insight into the anticancer effects of 1,25-VD in PCa, we have selected a vitamin D-resistant subclone, LNCaP-R, by growing the parental LNCaP-P cells in the presence of 100 nM 1,25-VD for 3 months. In agreement with the studies of vitamin D-resistant MCF-7 variants, LNCaP-R cells express comparable levels of VDR and RXR, but the transcriptional activity of VDR and cell growth inhibition appeared to be less sensitive to 1,25-VD. In addition, we applied Ketoconazole (an inhibitor of P450 enzymes), trichostatin A (TSA), (a histone deacetylase inhibitor), and TPA to enhance VDR transcriptional activity in LNCaP-R cell. Our data demonstrated that only TPA downstream signals could potentiate 1,25-VD-induced VDR transactivity and cell growth inhibition in LNCaP-R cells. Dissecting the mechanisms involved in the differential responsiveness of 1,25-VD using these two sublines might provide us valuable information for understanding the basic underlying mechanisms of vitamin D resistance and the actions of vitamin D.

2. Materials and methods

2.1. Materials and plasmids

1,25-VD and EB1089 were generous gifts of Dr. Lise Binderup from Leo Pharmaceutical Products (Ballerup, Denmark). Antibodies to VDR, p65, IRB-α, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody specific for phosphorylated-ERK1/2 (p-ERK1/2) was purchased from Cell Signaling Technology (Danvers, MA). TPA responsive element-luciferase (TRE-Luc) reporter construct was provided by Dr. Andrew M.-L. Chan from Mount Sinai School of Medicine, NY, NF-κB-Luc reporter construct was provided by Dr. Edward Schwarz from University of Rochester, and VDRE-Luc reporter construct was provided by Dr. Xianghuai Lu and Dr. Mark S. Nanes from Emory University School of Medicine, GA. The rCYP24A1-Luc construct has been described previously [8].

2.2. Cell culture and vitamin D-resistant subline selection

The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection. Cells were maintained in 10% FBS supplemented RPMI-1640 (Gibco-BRL, Carlsbad, CA) containing penicillin (100 IU/mL) and streptomycin (100 mg/mL). The vitamin D-resistant variant, LNCaP-R, was developed by plating LNCaP cells in culture medium containing 100 nM 1,25-VD at a density of 2×10^5 cells/100 mm plate. Every the other day, the medium was replaced with fresh medium containing 100 nM 1,25-VD and cells were passaged every 8 days. Continued culture of surviving cells in 100 nM 1,25-VD for over 3 months resulted in a stable cell line that grew equally well in the presence or absence of 1,25-VD. In parallel with the LNCaP-R cells, LNCaP-P was also developed by exposing to equivalent amounts of ethanol vehicle. After the two sublines were established, the cells were grown in the culture medium without ethanol or 1,25-VD, and the responsiveness of the LNCaP-R cells to 1,25-VD was assessed regularly. LNCaP-R cells remained resistant to 1,25-VD for 6 months after establishment.

2.3. Cell proliferation assay

Cells were seeded in 96-well plates at a density of 3000 cells/well. After overnight incubation, cells were treated with either ethanol vehicle (0.1%, v/v), 1,25-VD, EB1089, ketoconazole, TSA, or TPA at indicated concentrations. At the indicated time points, medium was replenished and cell proliferation was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma–Aldrich, St. Louis, MO) as described previously [25].

2.4. Flow cytometric analysis, real-time polymerase chain reaction (PCR), Western blot analysis, transient transfection, luciferase assay, and chromatin immunoprecipitation (ChIP) assay

The assays above were performed as described previously [25,26] or elsewhere [27]. The primers used in this study were described previously [8,25,27] and are listed in Table 1.

Table 1
Real-time PCR primer sequences.

Gene	Forward primer	Reverse primer
Protease M	5′-AAGGAGAAGCCAGGAGTC-3′	5′-TATTAAGCATCAGGGTCAGAG-3′
DRIP-205	5′-AAGCGGAAGAAGGCAGAC-3′	5′-GAGGAAGAGGAGGAAGAATGG-3′
SRC-1	5′-ATGGACAAATAATACAGTGACAG-3′	5′-AGGAGAAGGAGAAGAGTAAGG-3′
ALIEN	5′-CACGAGCCAAGATGTCTG-3′	5′-ACTCCGAATATAGGTCAATAGC-3′
LCOR	5′-CTCACCTCTGGACCTTAC-3′	5′-CTGCTCAGTAGTTCTTCAC-3′
hnRNPA1	5′-GGTGGCTATGGCGGTTCC-3′	5′-ACTTCTCTGGCTCTCCTCTCC-3′
hnRNPA2	5′-AGGAGGAAGAGGAGGATATGG-3′	5′-TCATTGGACCGTAGTTAGAAGG-3′
HDAC1	5′-GCCTAGTGCGGTGGTCTTAC-3′	5′-TCGTGTTCTGGTTAGTCATATTGG-3′
HDAC2	5′-GCTTGGAGGAGGTGGCTAC-3′	5′-ATTCTGGAGTGTTCTGGTTTGTC-3′
HDAC3	5′-TCTGGCTTCTGCTATGTC-3′	5′-AGGTGCTTGTAACTCTGG-3′
IκB-α	5′-CTCCACTCCATCCTGAAG-3′	5′-CCTCATCCTCACTCTCTG-3′
IκB-β	5′-TACTCCCGACACCAACCATACC-3′	5′-CCTCCTCACTCTCCTCTTCTTCC-3′

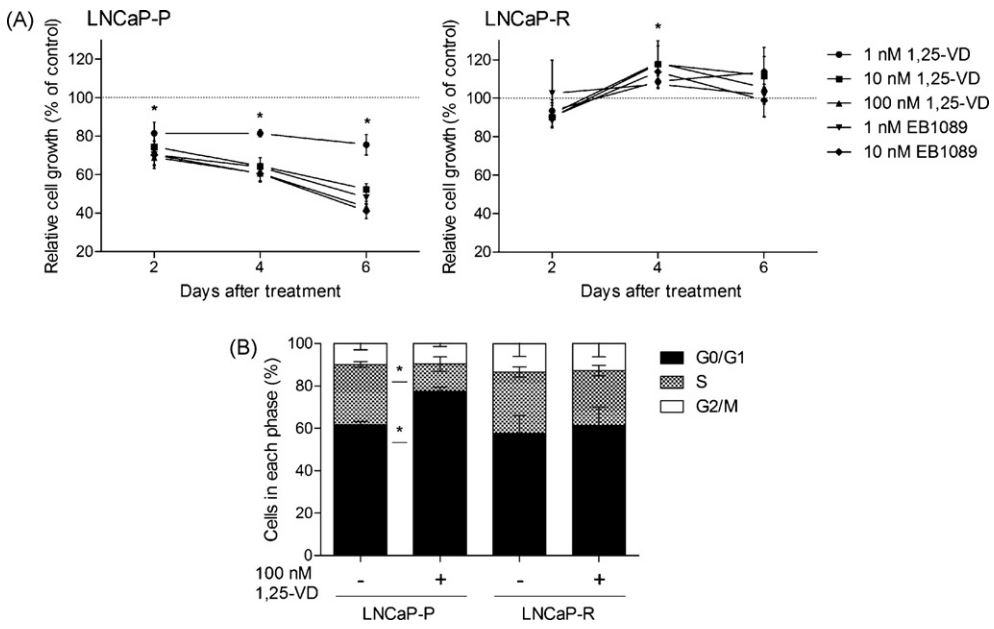

Fig. 1. The effects of 1,25-VD and EB1089 on human prostate cancer cell lines, LNCaP-P and LNCaP-R. (A) 1,25-VD inhibits the growth of LNCaP-P, but not its 1,25-VD-resistant variant, LNCaP-R cells. LNCaP-P and LNCaP-R cells were treated with ethanol vehicle or increasing concentrations of 1,25-VD and EB1089. At the indicated time points, cell growth was assessed by MTT assay and compared to vehicle-treated control. (B) 1,25-VD induces G0/G1 phase cell cycle accumulation in the LNCaP-P cells but not on LNCaP-R cells. LNCaP-P and LNCaP-R cells were treated with either ethanol vehicle or 100 nM 1,25-VD for 2 days. Cell cycle profiles were determined by flow cytometric analysis. $^*P < 0.05$.

2.5. Statistical analysis

The results are the mean ± SEM of values obtained from at least three experiments. ANOVA and Student's *t*-test were used to assess the statistical significance and the statistically significant difference was considered to be present at $P < 0.05$.

3. Results

3.1. Differential effects of 1,25-VD on cell growth and cell cycle of LNCaP-P and LNCaP-R cells

To understand the mechanism of the vitamin D resistance, we established a vitamin D-resistant cell line, LNCaP-R, by continued exposure of LNCaP cells to 100 nM 1,25-VD for 3 months. The surviving cells that are resistant to 1,25-VD were selected and expanded in normal culture medium. LNCaP-R cells remained resistant to 1,25-VD at least for 6 months after establishment.

The vitamin D anti-growth effects were then compared between parental LNCaP-P cells and LNCaP-R cells. As shown in Fig. 1, treatment of LNCaP-P cells with 1–100 nM 1,25-VD, and 1–10 nM EB1089, a synthetic vitamin D analogue not metabolized by CYP24A1, significantly suppressed cell growth (20–30% at day 2 and 20–60% at day 6, Fig. 1A, left). In contrast, the vitamin D antiproliferative effect was lost in LNCaP-R cells (Fig. 1A, right). Cell cycle profile analysis further demonstrated that 1,25-VD can promote G0/G1 growth arrest (from 61 ± 1.6% to 77 ± 2.1% at day 2) and S phase inhibition (from 29 ± 1.3% to 13 ± 3.4%) in LNCaP-P cells, but had no effect in LNCaP-R cells (Fig. 1B).

3.2. 1,25-VD-induced VDR transcriptional activity is reduced in LNCaP-R cells

Since 1,25-VD actions are mainly mediated through VDR, 1,25-VD/VDR-mediated transcription activity in LNCaP-P and LNCaP-R cells was then compared. As shown in Fig. 2A, the VDR transcriptional activities were determined by rCYP24A1-Luc and VDRE-Luc reporter activity, as well as VDR target genes expression in response to 1,25-VD. As expected, 1,25-VD-induced rCYP24A1-Luc and VDRE-Luc activities are significant reduced in LNCaP-R cells, and this loss of 1,25-VD responsiveness in LNCaP-R was further confirmed by a reduction of two VDR target genes, CYP24A1 and protease M, expression upon 1,25-VD treatment (Fig. 2B). Taken together, reduction of 1,25-VD/VDR-mediated transcriptional activity found in LNCaP-R cells might be responsible for loss of 1,25-VD antiproliferative effects.

3.3. Expression of VDR, RXRs, coregulators, and competitive VDRE-binding proteins are varied, but the DNA-binding abilities of VDR are similar between LNCaP-P and LNCaP-R cells

To further investigate the impairment of 1,25-VD-induced VDR transcriptional activity in LNCaP-R cells, VDR status, including variations in the VDR gene and the expression level were examined. As shown in Fig. 3A and B, VDR mRNA and protein expression levels in LNCaP-R are similar to parental cells, and treatment with 1,25-VD treatment did not influence its expression levels. In addition, there is no mutation, nor polymorphism in the VDR gene amplified from the complementary DNA of both cell lines (data not shown). The status of VDR-associated proteins that are critical for its transcriptional activity, including RXRs, VDR heterodimer partner, two coactivators, DRIP-205 and SRC-1, and two corepressors, NCoR1 and SMRT, were compared. To our surprise, the VDR-associated proteins express higher levels in LNCaP-R cells, whereas another two corepressors, ALIEN and LCOR (Fig. 3C), and competitive VDRE-binding proteins, hnRNPA1 and hnRNPA2 (Fig. 3D) show no difference in LNCaP-P and LNCaP-R cells. In summary, we found increasing or no difference on the expression levels of VDR-associated proteins, suggesting that these VDR-associated proteins are not the primary cause of vitamin D resistance.

1,25-VD actions are mediated by binding of VDR to the vitamin D response elements (VDREs) in the promoters of vitamin D-responsive genes, therefore, we compared the DNA-binding capacity of VDR to the CYP24A1 promoter between LNCaP-P and LNCaP-R cells using *in vivo* ChIP assays as described previously [26]. As shown in Fig. 3E, 1,25-VD induces VDR binding to the VDRE, to

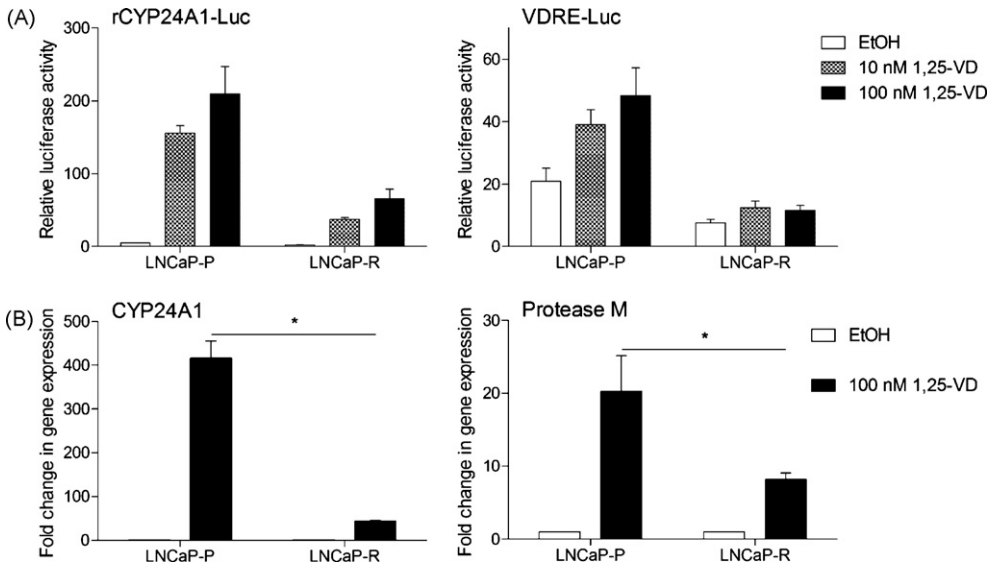

Fig. 2. Relative 1,25-VD-induced transcriptional activity of the VDR between LNCaP-P and LNCaP-R cells. (A) 1,25-VD-induced VDR transactivity is lower in LNCaP-R than in LNCaP-P cells. LNCaP-P and LNCaP-R cells were transiently transfected with 0.8 μg/well of rCYP24A1 and VDRE reporter constructs, and treated with ethanol vehicle, 10, or 100 nM 1,25-VD for 24 h. Reporter activities were measured via the dual-luciferase assay. (B) 1,25-VD-induced expression of VDR target genes is lower in LNCaP-R than in LNCaP-P cells. LNCaP-P and LNCaP-R cells were treated with ethanol vehicle or 100 nM 1,25-VD for 24 h. CYP24A1 and protease M expressions were analyzed by real-time PCR. Values represent the fold differences in gene expression relative to control. $^*P < 0.05$.

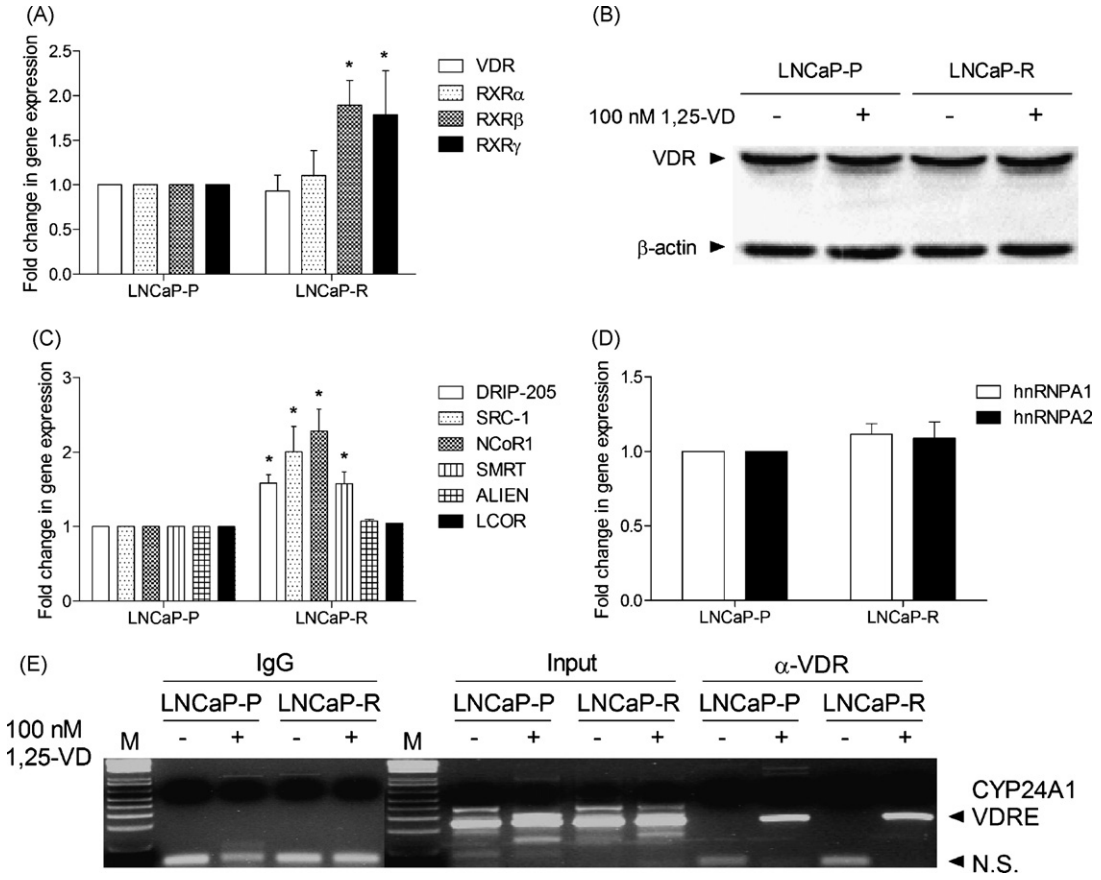

Fig. 3. Relative expression levels of VDR, RXRs, coregulators, and competitive VDRE-binding proteins between LNCaP-P and LNCaP-R cells. LNCaP-P and LNCaP-R were seeded at the same cell density and grown exponentially for 2 days. (A) VDR, RXRα, RXRβ, RXRγ, (C) DRIP-205, SRC-1, NCoR1, SMRT, ALIEN, LCOR, hnRNPA1, and (D) hnRNPA2 mRNA expressions were analyzed by real-time PCR. Values represent the fold differences in gene expression relative to LNCaP-P cells. (B) VDR protein expression. LNCaP-P and LNCaP-R were seeded at the same cell density for 1 day and then treated with either ethanol vehicle or 100 nM 1,25-VD for another 2 days. Cell lysates were analyzed for VDR content by Western blot. (E) 1,25-VD-stimulated VDR binding to human CYP24A1 VDRE is still intact in LNCaP-R cells. LNCaP-P and LNCaP-R cells were treated with ethanol vehicle or 100 nM 1,25-VD for 2 h, and then subjected to ChIP analysis using antibodies against VDR or IgG. Immunoprecipitated DNA was subjected to PCR amplification for CYP24A1 VDRE. M: DNA marker; N.S.: non-specific. $^*P < 0.05$.

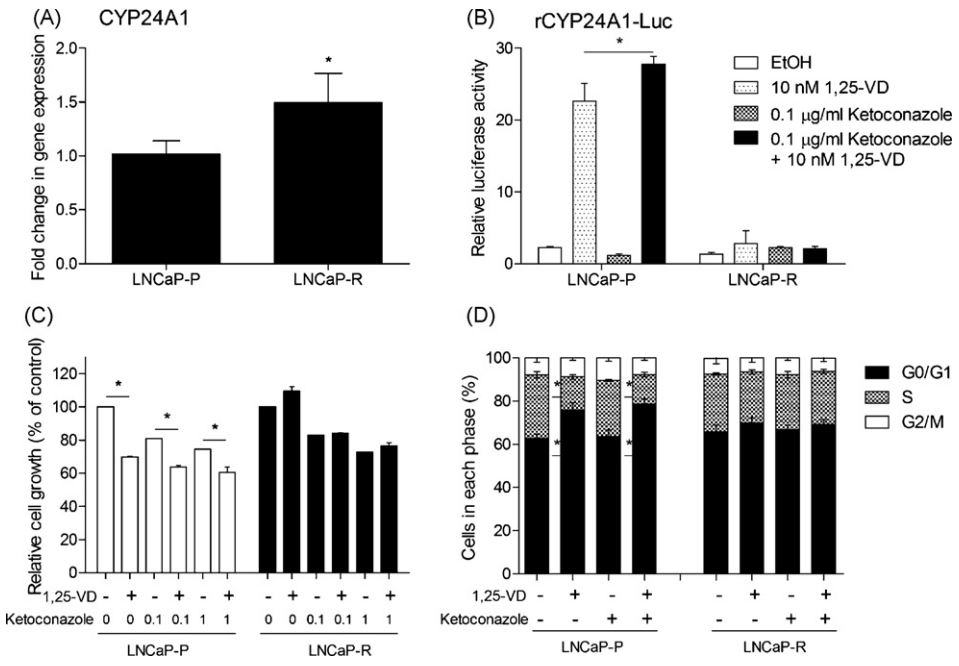

Fig. 4. LNCaP-R cells have higher CYP24A1 expression, but it is not the cause of 1,25-VD resistance. (A) Relative expression level of CYP24A1 between LNCaP-P and LNCaP-R cells. LNCaP-P and LNCaP-R were seeded at the same cell density and grown exponentially for 2 days. CYP24A1 mRNA expression was analyzed by real-time PCR. Values represent the fold differences in gene expression relative to LNCaP-P cells. (B) The effects of P450 inhibitor, Ketoconazole, on 1,25-VD-induced VDR transactivity. LNCaP-P and LNCaP-R cells were transiently transfected with 0.8 μg/well of rCYP24A1 reporter constructs and treated with ethanol vehicle, 10 nM 1,25-VD, 0.1 μg/ml Ketoconazole, or in combination for 24 h. Reporter activities were measured via the dual-luciferase assay. Inhibition of CYP24A1 does not sensitize LNCaP-R cells in response to 1,25-VD. LNCaP-P and LNCaP-R cells were treated with ethanol vehicle, 10 nM 1,25-VD, 0.1, 1 μg/ml Ketoconazole, or in combination, as indicated, for 6 days. Cell growth (C) was assessed by MTT assay and cell cycle profile (D) were determined by flow cytometric analysis. $^{*}P < 0.05$.

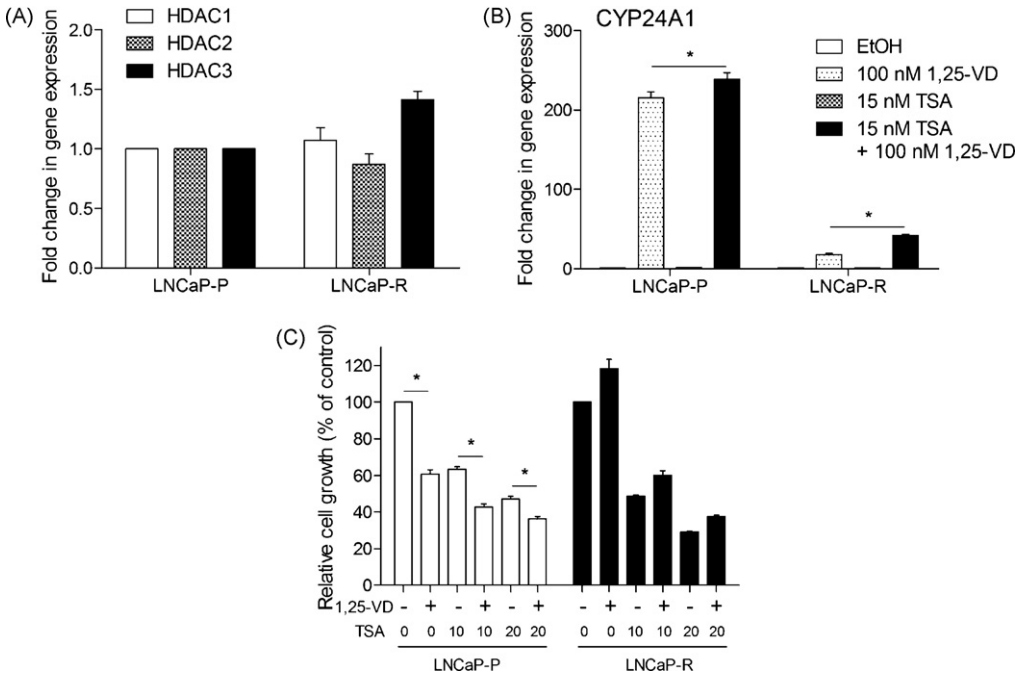

Fig. 5. Histone deacetylase inhibitor, TSA, potentiates 1,25-VD-induced VDR transactivity, but not the cell growth in LNCaP-R cells. (A) Relative expression levels of HDAC1, HDAC2, and HDAC3 between LNCaP-P and LNCaP-R cells. LNCaP-P and LNCaP-R were seeded at the same cell density and grown exponentially for 2 days. HDAC1, HDAC2, and HDAC3 mRNA expressions were analyzed by real-time PCR. Values represent the fold differences in gene expression relative to LNCaP-P cells. (B) TSA potentiates 1,25-VD-induced expression of VDR target gene, CYP24A1. LNCaP-P and LNCaP-R cells were treated with ethanol vehicle, 100 nM 1,25-VD, 15 nM TSA, or in combination for 24 h. CYP24A1 expressions were analyzed by real-time PCR. Values represent the fold differences in gene expression relative to control. (C) TSA does not sensitize LNCaP-R cells in response to 1,25-VD-induced growth inhibition. LNCaP-P and LNCaP-R cells were treated with ethanol vehicle, 10 nM 1,25-VD, 10, 20 nM TSA, or in combination, as indicated, for 6 days. Cell growth was assessed by MTT assay and compared to vehicle-treated control. $^{*}P < 0.05$.

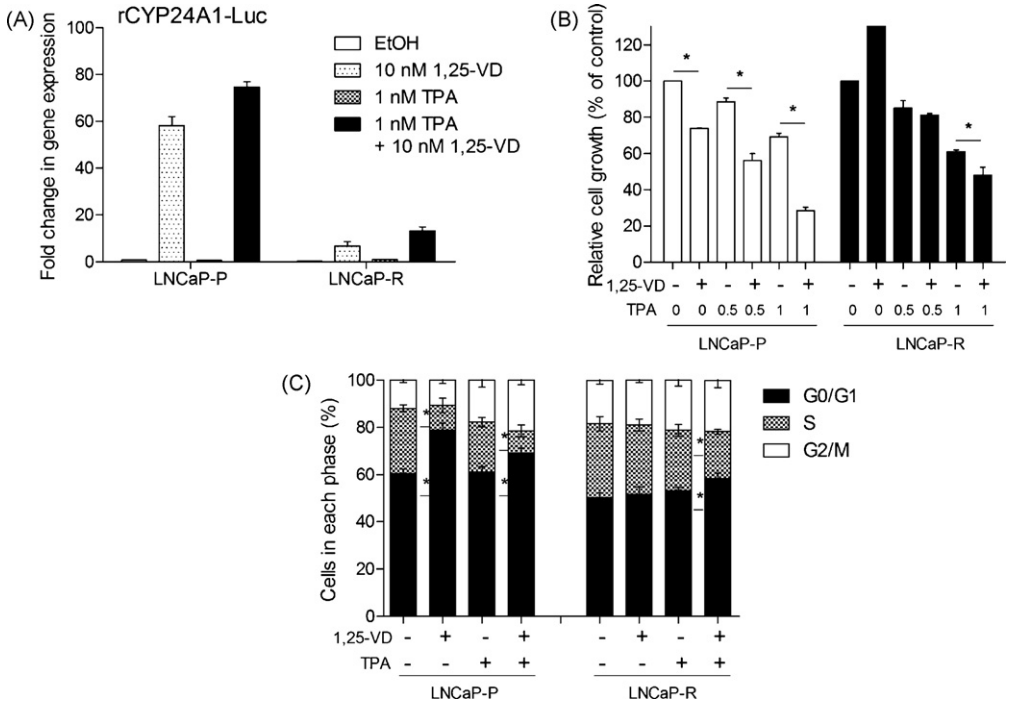

Fig. 6. TPA potentiates 1,25-VD-induced VDR transactivity and cell growth inhibition in LNCaP-R cells. (A) The effects of TPA on 1,25-VD-induced VDR transactivity determined by rCYP24A1-luc activity. Reporter activities were measured via the dual-luciferase assay. TPA sensitizes LNCaP-R cells in response to 1,25-VD-induced growth inhibition. LNCaP-P and LNCaP-R cells were treated with ethanol vehicle, 10 nM 1,25-VD, 0.5, 1 nM TPA, or in combination, as indicated. Cell growth (B) was assessed by MTT assay and cell cycle profiles (C) were determined by flow cytometric analysis. $^{*}P < 0.05$.

the same degree, in both cells. Overall, VDR gene, expression levels of VDR, expression levels of VDR, RXRs, coregulators, competitive VDRE-binding proteins, and the DNA-binding capacities are similar in these two cells.

3.4. LNCaP-R cells have higher CYP24A1 expression, but blocking 24-hydroxylase activity does not restore its 1,25-VD sensitivity

High levels of 24-hydroxylase have been shown in many vitamin D-nonresponsive cells, such as DU 145 [10]. To examine if the vitamin D-resistant phenotype in LNCaP-R cells results from the high levels of CYP24A1 expression, basal mRNA expression levels of CYP24A1 were compared between LNCaP-P and LNCaP-R cells. As shown in Fig. 4A, the basal level of CYP24A1 mRNA expression is 1.5-fold higher in LNCaP-R cells, suggesting the possibility that rapid breakdown of 1,25-VD by CYP24A1 might be the cause of the 1,25-VD resistance in LNCaP-R cells. Therefore, we tested whether blocking CYP24A1 activity by ketoconazole would restore 1,25-VD responsiveness. Cells were treated with ketoconazole, and 1,25-VD-induced VDR transcriptional activity and growth inhibition of LNCaP-R cells were measured. As shown in Fig. 4B–D, we found that 1,25-VD-induced VDR transactivity, growth inhibition, G0/G1 cell cycle accumulation, and S phase inhibition were potentiated by co-treatment with ketoconazole in LNCaP-P cells only, but not in LNCaP-R cells. Plus, LNCaP-R cells are also resistant to EB 1089, non-CYP450 metabolized vitamin D analogs (Fig. 1A, right). Taken together, these data suggest that the higher CYP24A1 expression did not contribute to 1,25-VD resistance in LNCaP-R cells.

3.5. Histone deacetylase inhibitor, TSA, potentiates 1,25-VD-induced VDR transactivity, but not the cell growth in LNCaP-R cells

Higher expression of two corepressors, NCoR1 and SMRT (Fig. 3C), implied that histone deacetylase activity might be altered and influence the function of VDR in LNCaP-R cells. NCoR1/SMRT has been shown to be responsible for recruitment by HDACs, which are required for transcriptional repression of many nuclear receptor target genes [28]. We found that the mRNA levels of three HDAC isoforms are variable between the LNCaP-P and LNCaP-R cells (Fig. 5A), suggesting that the HDAC activity might be regulated by association with the increased corepressors, NCoR1/SMRT, in LNCaP-R cells. We tested whether modulating VDR transcriptional activity (determined by CYP24A1 expression) by a HDAC inhibitor, trichostatin A (TSA), can restore the antiproliferative effects of 1,25-VD in LNCaP-R cells, and we found that the TSA promotes 1,25-VD-induced VDR transcriptional activity (Fig. 5B). However, treatment with TSA can suppress 1,25-VD-induced cell growth inhibition further, in LNCaP-P cells, but not in LNCaP-R cells (Fig. 5C), while treatment with 10–20 nM TSA alone can suppress cell growth by 40–50% in LNCaP-P and 50–70% in LNCaP-R (Fig. 5C). These results indicated that there are factors other than NCoR1/SMRT and HDAC contributing to the 1,25-VD resistance of LNCaP-R cells.

3.6. TPA potentiates 1,25-VD-induced VDR transactivity and cell growth inhibition in LNCaP-R cells

As shown in Fig. 3E, similar amounts of VDR bind to the VDRE in both cells, while LNCaP-R cells have a reduced 1,25-VD/VDR-mediated transcriptional activity. This suggests that the factors needed for optimizing VDR transactivity are impaired in LNCaP-R cells and account for the vitamin D resistance. TPA-induced signals have been known to collaborate with the 1,25-VD/VDR pathway to optimize VDR actions [23,29], so we tested the effects of TPA on VDR transcriptional activity and cell growth. As shown in Fig. 6, TPA could enhance VDR actions in both cells where it further enhances 1,25-VD mediated rCYP24A1-Luc reporter activity (Fig. 6A) and promotes 1,25-VD antiproliferative effects (Fig. 6B). TPA alone, at concentrations of 0.5–1 nM, suppressed growth of

both LNCaP-P and LNCaP-R cells by 20–40% (Fig. 6B), and this growth inhibition can be further suppressed by 1,25-VD treatment in both cells. TPA is a potent cell differentiating agent that induces cell arrest in G2–M [29–31]. As shown in Fig. 6C, 1,25-VD alone induced LNCaP-P cells G0–G1 arrest, and TPA alone induced G2–M arrest (from 12.0% to 17.8%). The combination of TPA and 1,25-VD induced G0–G1 and G2–M accumulation, whereas the S phase dramatically decreased from 27.6% to 9.2% in LNCaP-P cells. Similar effects, but to a lesser extent, were observed in LNCaP-R cells. We noticed that 1,25-VD induced a small but significant accumulation in G0–G1 phase (from 52% to 58%) and decrease in the S phase (from 26% to 20%) when co-treated with TPA in LNCaP-R cells (Fig. 6C), which is in agreement with the effects of 1,25-VD alone in LNCaP-P cells (Fig. 1B). Taken together, TPA treatment can partially restore the LNCaP-R sensitivity to 1,25-VD, suggesting that TPA-mediated signals are critical for optimizing 1,25-VD-mediated growth regulation.

3.7. Reduction of NF-κB pathway, a TPA-mediated downstream pathway, contributes to the 1,25-VD resistance of LNCaP-R cells

It has been demonstrated that TPA stimulates cell differentiation via the activation of several signaling kinases, including extracellular signal-regulated protein kinase (ERK)/p38 mitogen-activated protein kinase (MAPK) and inhibitory kappaB (IκB) kinase (IKK), and their downstream transcriptional factors, activator protein-1 (AP-1) and nuclear factor-kappaB (NF-κB) [32–35]. To compare the ERK and AP-1 activities between LNCaP-P and LNCaP-R cells under steady-state conditions, we performed Western blot analysis with phospho-ERK and AP-1-response element driven TRE-Luc activity. As shown in Fig. 7A, consistent with previous studies, we found that LNCaP cells displayed very low endogenous ERK1/2 activity [36] and there is no difference on downstream AP-1 activity by measuring the TRE-Luc activity, indicating that ERK/AP-1 pathway was not the cause for vitamin D resistance in our model. Interest-

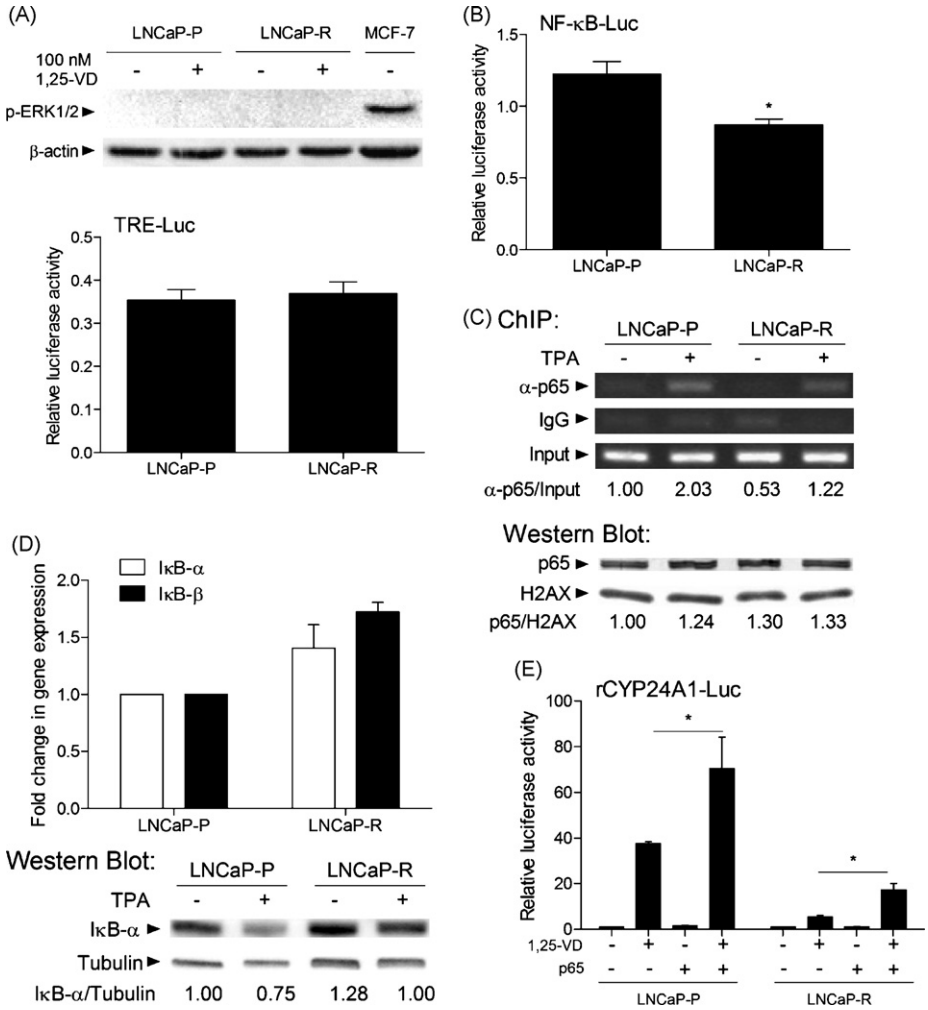

Fig. 7. Relative activities of TPA downstream ERK/AP-1 and NF-κB pathways between LNCaP-P and LNCaP-R cells, and their impact on the 1,25-VD resistance. (A) The relative ERK and AP-1 activities in LNCaP-P and LNCaP-R cells. Cells were seeded, then treated with either ethanol vehicle or 100 nM 1,25-VD for another 2 days. Phosphorylated-ERK1/2 levels were determined by Western blot. The relative AP-1 activities were assessed by measuring TRE-luc reporter activity. Determination of NF-κB activities by (B) NF-κB luc reporter activity and (C) DNA binding of p65 to NF-κB response element located in IL-8 promoter (upper panel) by ChIP assay. NF-κB-p65 protein expression (lower panel) was detected by Western blot, and H2AX was used as an internal control. Values represent the fold differences relative to untreated LNCaP-P cells. (D) Relative expression levels of IκB-α and -β. The mRNA expression of IκB-α and -β were analyzed by real-time PCR (upper panel), and the protein expression of cytoplasmic IκB-α was analyzed by Western Blot (lower panel) and tubulin was used as an internal control. Values represent the fold differences in gene expression relative to untreated LNCaP-P cells. (E) Overexpression of p65 potentiates 1,25-VD-induced VDR transactivity. LNCaP-P and LNCaP-R cells were transiently transfected with 0.4 μg/well of rCYP24A1 reporter construct, and 0.6 μg/well control vector or p65 expression plasmids as indicated. After transfection, cells were treated with ethanol vehicle or 10 nM 1,25-VD for another 24 h. Reporter activities were measured via the dual-luciferase assay. $^{*}P < 0.05$.

ingly, we found that LNCaP-R cells have lower NF-κB-dependent transcriptional activity as compared to LNCaP-P cells (Fig. 7B). To confirm this, we compared NF-κB-p65 DNA-binding capacity between LNCaP-P and LNCaP-R cells using *in vivo* ChIP assay. As shown in Fig. 7C, TPA induced a 2-fold increase of p65 binding to the NF-κB responsive element which is in accordance with the luciferase assays; in contrast, LNCaP-R cells had only 50% of p65 DNA-binding capacity compared to LNCaP-P cells in either basal or under TPA stimulation. To further characterize the cause of the lower p65 DNA-binding capacity, we examined the nuclear p65 levels in LNCaP-P and LNCaP-R cells. H2AX, a variant of histone H2A, was used as an internal control to verify equal nuclear protein loading. As expected, TPA induced the nuclear enrichment of p65 (Fig. 7C). Interestingly, we found that LNCaP-R cells had higher nuclear p65 protein levels, but displayed less response to TPA stimulation than LNCaP-P cells (1.02-fold TPA-induced p65 nuclear enrichment in LNCaP-R cells *vs.* 1.24-fold in LNCaP-P cells).

The biological activity of the transcriptional factor NF-κB is controlled mainly by the IκB [37]. As shown in Fig. 7D, we found that the mRNA expression levels of IκB-α and -β, as well as the expression level of IκB-α protein were higher in LNCaP-R cells. Therefore, it is likely that the lower NF-κB activity in LNCaP-R cells is attributed to the higher IκB expression. To prove that lower NF-κB activity in LNCaP-R is one major contributor to 1,25-VD resistance, we restored NF-κB activity by overexpression of NF-κB subunit p65 to LNCaP-R, and examined whether increased NF-κB activity could restore VDR transcriptional activity. As shown in Fig. 7E, overexpression of p65 increased 1,25-VD-induced VDR transactivity by 87% in LNCaP-P cells and 217% in LNCaP-R cells. This much stronger VDR transactivation promoted by overexpression of p65 in LNCaP-R cells, as compared to LNCaP-P cells, further supports that NF-κB signaling is critical for vitamin D sensitivity and impairment of NF-κB signal pathway contributes to vitamin D resistance and disease progression.

4. Discussion

To investigate the mechanisms controlling the development of 1,25-VD resistance in PCa cells, we established a new vitamin D-resistant human PCa cell line, LNCaP-R, by long-term exposure of LNCaP cells to high concentrations of 1,25-VD. The findings observed in our study, including cross-resistance to 1,25-VD analog, comparable to VDR expression level and VDRE-binding ability, but lower VDR transcriptional activity, are in agreement with previous results found in the vitamin D-resistant MCF-7 variants [22,24]. Our data showed that LNCaP-P cell growth can be suppressed around 20–55% by 1–100 nM 1,25-VD (Fig. 1A, left). However, there were reports that lower concentrations of 1,25-VD may stimulate the growth of some neoplastic cell lines [38–40], which was also observed in our LNCaP-R vitamin D-resistant subline (Fig. 1A, right).

CYP24A1 is a well-known VDR targeted gene in which 1,25-VD can induce CYP24A1 to suppress production of 1,25-VD [41–43]. CYP24A1 has been described as a candidate oncogene, whose DNA amplification and overexpression might give a growth advantage to cancer cells and abrogate vitamin D-mediated growth control [44]. Furthermore, genistein, ketoconazole, and liarozole can act synergistically with 1,25-VD to inhibit DU 145 cell growth by inhibiting the activity of CYP24A1 or its expression [10,45,46], suggesting that CYP24A1 overexpression might be one of the mechanisms causing vitamin D resistance. However, treatment with ketoconazole does not potentiate the 1,25-VD-induced VDR transactivity and growth inhibition in LNCaP-R cells (Fig. 4B and C). In addition, because the side-chain double-bond structure of EB1089 is hydroxylated in distal C26 and C26a sites, the metabolism of EB1089 might not involve CYP24A1 [47]. EB1089 has been shown to be more potent in the cancer cell growth inhibition than 1,25-VD both *in vivo* and *in vitro* [48–51]. As shown in Fig. 1A, EB1089, at 1 nM, exerts similar growth inhibition with 1,25-VD at 100 nM in LNCaP-P cells, whereas LNCaP-R cells are still resistant to EB1089 (Fig. 1A, right). This, further supports that higher CYP24A1 expression in LNCaP-R is not the cause of 1,25-VD resistance in our model.

Steroid hormone receptor coactivators and corepressors regulate the initiation of transcription by altering the level of histone acetylation and deacetylation [52]. In the absence of 1,25-VD, VDR associates with a gene repression complex containing corepressors, such as NCoR1, SMRT, ALIEN, and LCOR, and HDACs, thereby maintaining a closed chromatin structure and suppressing transcription of target genes [53–55]. Upon the 1,25-VD ligand binding, the conformational change of VDR promotes the association with coactivator complexes, such as SRC-1, GRIP-1, and DRIPs, and enhances transcription initiation by chromatin acetylation [56,57]. Thus, the altered activity and expression of VDR cofactors may in turn influence HDAC activity and thereby account for different antiproliferative responses to 1,25-VD. It has been found that the HDAC inhibitor, TSA, synergistically cooperated with 1,25-VD in growth inhibition of prostate cancer cells [58,59]. Furthermore, VDR corepressors, NCoR1 and SMRT, are frequently elevated in primary breast and PCa tumors, and are often associated with reduced sensitivity to 1,25-VD [60,61]. Targeted knock down of NCoR1 and SMRT using siRNA did enhance 1,25-VD-induced gene expression and reverse the 1,25-VD insensitivity [8,61]. Consistent with previous studies, we found that the expression levels of VDR corepressors, NCoR1 and SMRT, are elevated in vitamin D-resistant LNCaP-R cells, but its coactivators, SRC-1 and DRIP-205, and its heterodimeric partner, RXRβ and RXRγ, are also increased (Fig. 3A and C). Although TSA acted synergistically with 1,25-VD to induce VDR transcriptional activity and inhibit the growth of LNCaP-P cells, it does not restore the antiproliferative action of 1,25-VD in LNCaP-R cells (Fig. 5A and C), indicating that altered levels of acetylation/deacetylation and expressions of VDR cofactors are not key factors in our vitamin D-resistant cell model.

Even though we and others have demonstrated that TPA can potentiate VDR transactivity and restore 1,25-VD-mediated growth inhibition in LNCaP-R cells, downstream targets involved in this resensitization have not yet been identified. TPA can activate many kinases of the intracellular signaling network, such as protein kinase C (PKC), MAPK, and IKK, thereby activating a battery of transcription factors including AP-1 and NF-κB [62,63]. AP-1 is a transcriptional factor complex composed of proteins of the Fos and Jun proto-oncogene families, which need dimerization to promote the complex binding to the AP-1 recognition site. It has been reported that TPA can induce MAPK activity and MAPK-mediated phosphorylation is required for activation of the Fos and Jun families. From the pharmacological inhibitors studies, TPA-induced activation of AP-1 was abrogated by treatments with p38 MAP kinase inhibitor, SB203580 and c-Jun NH2-terminal kinase (JNK) specific inhibitor SP600125, but not MEK inhibitor U0126, suggesting that TPA induces AP-1 activation via the p38 and JNK signaling pathway [32,64,65]. In resting cells, NF-κB resides in the cytoplasm by forming an inactive complex with IκB. Upon stimuli, IκB gets phosphorylated by IKK and subsequently degraded by proteasome, thereby leaving NF-κB free to translocate to the nucleus and activate transcription. TPA can stimulate IKKα/β activity, phosphorylation and degradation of IκB-α, nuclear translocation of p65, DNA binding of NF-κB, and expression of NF-κB target genes [66]. Although AP-1 and NF-κB are regulated by different mechanisms, a number of reports have shown that the AP-1 activity is strikingly enhanced when NF-κB subunits are present and vice versa [67]. We found that there is undetectable endogenous ERK1/2 activity and no difference in downstream AP-1 activity comparing LNCaP-P and LNCaP-R cells (Fig. 7A). Intriguingly, in agreement with higher

IκB-α and -β expression levels, we found that LNCaP-R cells have lower NF-κB activity (Fig. 7B–D). Furthermore, we demonstrated that 1,25-VD-induced VDR transactivity was increased by overexpression of p65 (Fig. 7E) in both LNCaP-P and LNCaP-R cells, suggesting that NF-κB signaling might be critical for VDR transcriptional activity and the growth inhibition of 1,25-VD.

NF-κB-regulated genes play important roles in several physiological processes, such as inflammation, immunity, cell growth, cell survival, and development. Vitamin D and its various synthetic analogues have been widely used in the treatment of inflammatory and hyperproliferative disorders [68,69]. Although exactly how vitamin D and its metabolites exert these functions remains uncertain, several studies have suggested that at least part of the common mechanism is to increase or stabilize IκB-α, block p65 nuclear translocation, or disrupt p65/p50 binding to the NF-κB sites in the gene promoter, thus down-regulating proinflammatory chemokine production or inducing cell differentiation and growth arrest [70–73]. On the other hand, very few studies have addressed the effects of NF-κB on vitamin D actions. It has been shown that TNF-α-activated NF-κB can be integrated into the VDR transcription complex [74], however, this integration interferes with the VDR complex binding to the VDRE of the osteocalcin promoter in osteoblastic ROS 17/2.8 cells by disrupting the interaction between VDR and coactivators, such as SRC-1. Deletion analysis of p65 revealed that the N-terminus of p65 and a midmolecular region are both needed for the p65 inhibition on 1,25-VD actions [74]. The cause of the discrepancy between their and our results is not currently known; it is possible that the effects of p65 on VDR transactivity may vary depending on the cell context or the phosphorylation status of NF-κB after 1,25-VD, TPA, or TNF-α treatment. In contrast to the TNF-α-mediated inhibitory effects of p65 on VDR activity, TPA has also been shown to synergize with 1,25-VD to stimulate IκB-α phosphorylation, NF-κB nuclear translocation, and monocytic differentiation in NB4 leukemia cells [75]. In addition, it has been known that the phosphorylation status of p65 determines whether it associates with either CREB binding protein (CBP)/p300 or HDAC1 to repress or activate transcription [76], thus upstream kinases of the intracellular signaling network and the cell microenvironment might contribute to the differential NF-κB effects on 1,25-VD actions. Finally, NF-κB has been reported to have pro- and anti-apoptotic effects depending on the specific cell type and the type of stimulus. In T cells, the induction of apoptosis by glucocorticoids could be facilitated by inhibition of NF-κB, whereas apoptosis induced by phorbol ester, such as TPA and ionomycin, required activation of NF-κB and consequent upregulation of Fas-ligand [77]. Although it has been shown that 1,25-VD is not likely to induce PCa cell apoptosis through extrinsic pathways triggered by cytokine receptors [78], accumulating evidence suggested that induction of NF-κB activity can sensitize some cells to apoptosis-inducing agents that act through or cooperate with the NF-κB-dependent regulation of the cell cycle and death-promoting genes, such as p53. Overexpression of c-Rel in HeLa cells causes cell cycle arrest by a decrease in cyclin-dependent kinase (CDK) 2 kinase activity and increased levels of the tumor suppressor p53 and the CDK inhibitor p21 [79]. These observations are in accordance with the effects of 1,25-VD found in PCa cells [80]. Interestingly, some apoptosis-resistant T cells also have a deficiency in NF-κB-mediated gene transcription [81]. Therefore, detailed mechanisms of how NF-κB integrates into 1,25-VD actions and its role in vitamin D resistance need to be further investigated.

Here, we generated PCa subclones, LNCaP-P and LNCaP-R, as a model for studying the mechanisms of vitamin D resistance. Intriguingly, VDR is neither mutated nor lost/reduced in expression, and many VDR coactivators and corepressors are increased; yet the transcriptional activity of VDR is reduced in vitamin D-resistant LNCaP-R cells. Our results suggest that defects in a diverse range of intracellular signals, such as TPA/NF-κB signaling, might alter posttranslational modifications of downstream targets or VDR complexes, thus weaken the 1,25-VD-induced transcriptional activity and contribute to reduced vitamin D sensitivity. This study provides a valuable tool and information in identifying the basic mechanisms underlying the development of vitamin D resistance, and may lead to the design of better combination therapies in the management of advanced PCa.

Acknowledgments

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