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14. ABSTRACT

The original purpose of this research, as proposed in the statement of work, was to determine the mechanism by which prostate cancer (PCa) cells become resistant to the anti-tumor activity of vitamin D. The proposal focused on a PCa-specific deficiency in a key vitamin D metabolizing enzyme, 1α-hydroxylase (1αOH). We encountered unforeseen difficulties with one of the key techniques in the original proposal. Therefore we decided to focus on vitamin D target genes, whose expression would be effected downstream of 1αOH bioactivation of vitamin D. Using normal human prostatic epithelial cells and prostate cancer cell lines, we examined the role of map kinase phosphatase 5 (MKP5), a recently discovered target gene of vitamin D, in mediating anti-tumor activities. Upregulation of MKP5 mRNA by 1,25-dihydroxyvitamin D_3 (1,25D) was found to be dependent upon the vitamin D receptor (VDR) and the MKP5 promoter contains a putative positive vitamin D response element that associates with the VDR upon treatment with 1,25D. MKP5 dephosphorylates/inactivates the stress activated protein kinase p38. Interestingly, in the prostate cancer cell lines LNCaP, PC-3 and DU 145, 1,25D did not upregulate MKP5 or inactivate p38. Treatment of prostate cells with 1,25D inhibited basal and stimulated phosphorylation of p38, and MKP5-siRNA blocked this effect. We next investigated the effect of 1,25D on expression of interleukin-6 (IL-6), a downstream target of activated p38. IL-6 is an inflammatory cytokine and inflammation has been implicated in the initiation and promotion of prostate cancer. We found that 1,25D inhibited both UV- and tumor necrosis factor (TNF)-α-stimulated IL-6 production in normal cells via p38 inhibition. Consistent with inhibition of p38, 1,25D decreased UV-stimulated IL-6 mRNA stabilization. These studies reveal MKP5 as the mediator of inhibition of p38 and IL-6 expression by vitamin D in normal prostate cells, activities that are relevant to cancer prevention. Absence of MKP5 induction in prostate cancer cell lines suggests that selective pressure may eliminate key tumor suppressor functions of vitamin D during cancer progression. Our findings point to a new molecular mechanism for prostate cancer resistance to anti-tumor activity of vitamin D.

15. SUBJECT TERMS
Vitamin D, cancer prevention, stress-activated protein kinases, interleukin-6

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Introduction

The original purpose of this research, as proposed in the statement of work, was to determine the mechanism by which prostate cancer (PCa) cells become resistant to the anti-tumor activity of vitamin D. The experiments in the proposal were focused upon one particular aspect of PCa resistance to vitamin D, decreased enzymatic activity of vitamin D 1α-25-hydroxylase (1αOH). 1αOH is the enzyme that converts vitamin D to its active form, 1,25-dihydroxyvitamin D (1,25D). Since the ability of 1αOH to synthesize 1,25D has been shown to be impaired in PCa compared to normal prostate (1), we hypothesized that this is a key mechanism by which PCa escapes growth control by 1,25D.

Body/Results

The key assay necessary for us to investigate our hypothesis was the 1αOH enzymatic activity assay. Despite multiple attempts to troubleshoot the assay, I was unable to consistently measure 1αOH enzymatic activity. Since I was not able to measure 1αOH enzymatic activity itself, we decided to focus on vitamin D target genes, whose expression would be effected downstream of 1αOH bioactivation of vitamin D. We investigated a newly discovered molecular target of vitamin D, MAP kinase phosphatase 5 (MKP5), whose expression is dose-dependently upregulated by vitamin D. Furthermore, MKP5 expression appears to be diminished or absent in PCa cells. Although the experiments performed during this past year differ slightly from the proposed experiments, they address the original purpose of the work, which was to elucidate the mechanisms by which PCa cells become resistant to the anti-tumor activity of vitamin D. This shift in the focus of our experiments proved to be advantageous and I have submitted a manuscript to Cancer Research reporting the significance of our findings (appendix A).

Using cDNA microarrays, we had recently identified a new vitamin D-responsive gene, MAP kinase phosphatase 5 (MKP5) (2). The mRNA expression of MKP5, also known as dual specificity phosphatase 10 (DUSP10), is consistently increased by 1,25D treatment of primary cultures of prostatic epithelial cells (2). As a member of the dual specificity MKP (DS-MKP) family of proteins that dephosphorylate mitogen activated protein kinases (MAPKs), MKP5 dephosphorylates p38 and JNK, but not ERK (3,4). The potential ability of vitamin D to inhibit p38 through MKP5 is of interest to PCa prevention since p38 is activated by oxidative stress, hypoxia and inflammation, all of which contribute to PCa development (5-8). In particular, inflammation plays a causal role in the progression of many cancers including liver, bladder and gastric cancers (9) and a similar role for inflammation in the development of PCa is now emerging (8,10,11). The strongest evidence linking inflammation to PCa is from the recent findings that show regular administration of non-steroidal anti-inflammatory drugs (NSAIDS) significantly decreases PCa risk in older men by 60-80% (8,12). Also, men with chronic and/or acute inflammation of the prostate, either in the form of prostatitis or a sexually transmitted disease, have an increased risk of developing PCa (7,13).

Inhibitors of p38 are classically anti-inflammatory, suggesting that some of the activities attributed to vitamin D, including prostate cancer prevention, may be a result of p38 inhibition and decreased inflammation. One of the downstream consequences of p38 protein kinase pathway activation is an increase in pro-inflammatory cytokine production in order to amplify the inflammatory response (14,15). Interleukin-6 (IL-6) is a p38-regulated pleiotropic cytokine that has been historically associated with PCa (16,17). Elevated levels of IL-6 are found in the serum of PCa patients and primary PCa tumors over-express IL-6 (16,18). IL-6 is also involved in the progression of PCa to androgen-independent PCa because it can facilitate androgen receptor signaling in the absence of androgens (17,19,20).

Since there is increasing evidence linking inflammation to PCa development, our studies focused on characterizing regulation of IL-6 production via p38 inhibition by 1,25D as a potentially significant cancer prevention activity. Using primary epithelial cell cultures (E-PZ) derived from the normal peripheral zone [the major site of origin of prostatic adenocarcinomas(21)] as our model system, we characterized MKP5 induction by 1,25D and revealed MKP5 as the mediator of p38 kinase inhibition and decreased IL-6 production by 1,25D in normal prostatic epithelial cells. We also compared the ability of 1,25D to regulate MKP5 in prostate cancer-derived cells to the normal prostatic cells.

The materials, methods, results and figures are detailed in our submitted manuscript "Inhibition of p38 by Vitamin D reduces Interleukin-6 production in normal prostate cells via MAP Kinase Phosphatase 5: Implications for prostate cancer prevention by Vitamin D" (Appendix A.)

Key Research Accomplishments

The major findings of my research are:

1. 1,25D selectively increases MKP5 mRNA expression in primary cultures of normal prostatic epithelial cells and not in prostate cancer cells.

2. Upregulation of MKP5 mRNA by 1,25D is VDR dependent and MKP5 promoter contains a putative VDRE that associates with VDR upon 1,25D treatment.

3. MKP5-mediated p38 inactivation by 1,25D occurs in E-PZ cells and not in PCa cell lines.

4. MKP5 mediates decreased IL-6 production in E-PZ cells by 1,25D.

5. 1,25D inhibits TNFα-stimulated p38 activation and IL-6 production.

Reportable Outcomes

- Primary Author Manuscripts:

  Nonn L, Peng LH, Feldman, D, Peehl DM. Inhibition of p38 by Vitamin D reduces Interleukin-6 production in normal prostate cells via MAP Kinase Phosphatase 5: Implications for prostate cancer prevention by Vitamin D. *Cancer Res* submitted October 2005

- Contributing Author Manuscripts:

  Moreno J, Krishan AV, Swami S**, Nonn L**, Peehl DM, Feldman D. Regulation of prostaglandin metabolism by calcitriol (Vitamin D) attenuates growth stimulation in prostate cancer cells. *Cancer Res* 65(17):7917-7925, 2005.

- Awards and Meetings:

November 2004, Attended American Association for Cancer Research meeting “Basic, Translational, and Clinical Advances in Prostate Cancer” and received Scholar-in-training travel supplement award

- Funding:

  June 2005, Applied for NIH award (#1 RO1 CA 120254-01) based on work supported by this DOD award, “Prostate Cancer Prevention by Vitamin D via p38 Protein Kinase Inhibition”. Score is not within funding limits. Plan to resubmit in March 2006

Conclusions

We found that, consistent with increased levels of MKP5 mRNA, 1,25D inhibited p38 phosphorylation in normal E-PZ cells indicative of increased MKP5 protein and activity levels. MKP5-siRNA blocked p38 inactivation by 1,25D, which further demonstrated that MKP5 mediates p38 inactivation by 1,25D. The ability of 1,25D to inactivate p38 led us to examine the regulation of IL-6, which is downstream of p38 activation (22), by 1,25D. Suppression of UV-stimulated IL-6 production by 1,25D was previously demonstrated in keratinocytes (23) and we suspected that similar activity could be mediated by MKP5 in normal prostatic epithelial cells. Using MKP5-siRNA, we demonstrated that 1,25D inhibited UV-stimulated p38 activity and IL-6 production in a MKP5-dependent manner in E-PZ cells. Induction of IL-6 by a more physiologically relevant stress, TNFα, was similarly inhibited by 1,25D.

Another important finding from our study was that 1,25D upregulated MKP5 only in the cells derived from normal prostatic epithelium, including pRNS-1-1 cells. Although pRNS-1-1 cells originated from normal prostatic epithelial cells (E-PZ), they were immortalized by SV40 TAg and are no longer sensitive to growth-inhibition by 1,25D (24,25). Yet, pRNS-1-1 cells retain functional VDRs and upregulate vitamin D 24-hydroxylase, a classic vitamin D target gene, in response to 1,25D. Stromal cells (F-PZ) derived from normal prostatic tissue did not induce MKP5 when treated with 1,25D, although these cells express VDR and are growth-inhibited by 1,25D. PCa cell lines also are growth inhibited by 1,25D (26) while MKP5 was not upregulated in these cell lines. Together these observations demonstrate that 1) induction of MKP5 by 1,25D is specific to normal prostatic epithelial cells and does not occur in normal prostatic stromal cells, 2) induction of MKP5 is independent of growth inhibition by 1,25D, and 3) lack of 1,25D-induced MKP5 in PCa cell lines is not a result of immortalization per se since immortalized pRNS-1-1 cells upregulate MKP5 in response to 1,25D. From the qRT-PCR data, it also appears that the basal levels of MKP5 are lower in the PCa cell lines compared to normal prostatic epithelial cells. Since the established PCa cell lines have low levels of MKP5 and are unable to induce MKP5 in response to 1,25D, it is tempting to speculate that loss of MKP5 may occur in PCa as the result of selective pressure to eliminate tumor suppressor activity of MKP5 and/or 1,25D. A number of MKP family members have been suggested to be tumor-suppressors. In PCa MKP1 has been shown to be downregulated (27). Candidate MKP tumor suppressors in other malignancies include MKP7, which is frequently deleted in lymphoblastic leukemia (28), MKP3, hypermethylated or deleted in pancreatic cancer (29,30), and MKP2, which is deleted in breast carcinoma (31).

It is becoming apparent that inflammation, both chronic and acute, contributes to PCa development (11). If inflammation is a significant risk factor for PCa, then PCa prevention will best be achieved with agents, like vitamin D, that inhibit inflammation and/or decrease the cellular stress response that accompanies inflammation. Our study shows that MKP5 is a mediator of anti-inflammatory effects of 1,25D and suggests that vitamin D may play a

significant role in PCa prevention by facilitating p38 inhibition and reduced IL-6 production in normal human prostatic epithelial cells.

During this next year of research on this project, we would like to utilize MKP5 expression as a tool to examine the consequences of reduced 1αOH expression in PCa. We discovered during the course of our research that although MKP5 expression is diminished in the established PCa cell lines and primary epithelial cultures derived from high grade prostate cancer, primary epithelial cultures derived from low grade prostate cancer still maintain the ability to upregulate MKP5 in response to 1,25D treatment. Our lab and others have shown evidence of reduced 1αOH activity in low grade PCa. MKP5 mRNA expression is upregulated in a dose-dependent manner by 1,25D. 1,25D is the active form of vitamin D and product of 1αOH activity. We will measure MKP5 mRNA after dosing primary cultures of prostate cells derived from normal (E-PZ) and malignant tissue (E-CA) with 25D, the inactive precursor to 1,25D and substrate for 1αOH. Since E-CA cells have decreased 1αOH activity compared to normal prostate cells, the E-CA cells will convert less of the 25D to 1,25D. Decreased intracellular concentrations of active 1,25D in the PCa cells should cause attenuated upregulation of MKP5 mRNA. If MKP5 expression does prove to be an accurate readout of 1αOH activity in the prostate cells, we can use it as surrogate marker of 1αOH activity to address more of the experiments in our proposal.

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Appendices

(A) **Nonn L**, Peng LH, Feldman, D, Peehl DM. Inhibition of p38 by Vitamin D reduces Interleukin-6 production in normal prostate cells via MAP Kinase Phosphatase 5: Implications for prostate cancer prevention by Vitamin D. *Cancer Res* submitted October 2005

(B) Curriculum Vitae

Inhibition of p38 by Vitamin D reduces Interleukin-6 production in normal prostate cells via MAP Kinase Phosphatase 5: Implications for prostate cancer prevention by Vitamin D

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Running title: Vitamin D inhibition of p38 via MKP5 in normal prostate

Keywords: prostate cancer, vitamin D, MKP5, p38, IL-6

ABSTRACT

Although numerous studies have implicated vitamin D in preventing prostate cancer, the underlying mechanism(s) remain unclear. Using normal human prostatic epithelial cells, we examined the role of map kinase phosphatase 5 (MKP5), a recently discovered target gene of vitamin D, in mediating cancer preventive activities. Upregulation of MKP5 mRNA by 1,25-dihydroxyvitamin D_3 (1,25D) was found to be dependent upon the vitamin D receptor (VDR) and the MKP5 promoter contains a putative positive vitamin D response element that associates with the VDR upon treatment with 1,25D. MKP5 dephosphorylates/inactivates the stress activated protein kinase p38. Treatment of prostate cells with 1,25D inhibited basal and stimulated phosphorylation of p38, and MKP5-siRNA blocked this effect. We next investigated the effect of 1,25D on expression of interleukin-6 (IL-6), a downstream target of activated p38. IL-6 is an inflammatory cytokine and inflammation has been implicated in the initiation and promotion of prostate cancer. We found that 1,25D inhibited both UV- and tumor necrosis factor (TNF)-α-stimulated IL-6 production in normal cells via p38 inhibition. Consistent with inhibition of p38, 1,25D decreased UV-stimulated IL-6 mRNA stabilization. Interestingly, in the prostate cancer cell lines LNCaP, PC-3 and DU 145, 1,25D did not upregulate MKP5 or inactivate p38. These studies reveal MKP5 as the mediator of inhibition of p38 and IL-6 expression by vitamin D in normal prostate cells, activities that are relevant to cancer prevention. Absence of MKP5 induction in prostate cancer cell lines suggests that selective pressure may eliminate key tumor suppressor functions of vitamin D during cancer progression.

INTRODUCTION

Prostate cancer (PCa) is unique among malignancies in that it generally grows very slowly, likely for decades, before symptoms arise and a diagnosis is finally made. Seemingly the latency observed in PCa should provide a long window of opportunity for intervention by chemopreventive agents. Laboratory and epidemiology studies have shown a potential role for vitamin D in the prevention of PCa. Evidence supporting a role for vitamin D in PCa prevention began with studies that linked reduced serum levels of vitamin D metabolites to PCa incidence. Corder et al. were the first to find that low serum levels of 1,25-dihydroxyvitamin D_3 (1,25D), the active vitamin D metabolite, were associated with increased risk of prostate cancer in older men (1). Decreased serum levels of 25-hydroxyvitamin D_3 (25D), the circulating precursor to 1,25D, also correlate with an increased risk of PCa (2, 3). The latter finding has become more compelling since the discovery that prostate cells are not only sensitive to circulating 1,25D, but can also synthesize 1,25D from circulating 25D. Conversion of 25D to active 1,25D by vitamin D 1α-hydroxylase (4, 5)occurs within the normal prostate and suggests that local production of 1,25D may play a critical role in maintaining normal growth and differentiation.

Studies demonstrating that 1,25D inhibits the growth of primary cultures of prostate cells, established prostate cancer cell lines, and prostate xenograft tumors provide direct evidence for anti-cancer activity of vitamin D (6-9). However, mechanisms other than growth inhibition may be responsible for the prevention of PCa by vitamin D.

Using cDNA microarrays, we recently identified a new vitamin D-responsive gene, MAP kinase phosphatase 5 (MKP5) (10). The mRNA expression of MKP5, also known as dual specificity phosphatase 10 (DUSP10), is consistently increased by 1,25D treatment of primary cultures of prostatic epithelial cells (10). As a member of the dual specificity MKP (DS-MKP) family of proteins that dephosphorylate mitogen activated protein kinases (MAPKs), MKP5 dephosphorylates p38 and JNK, but not ERK (11, 12).

The potential ability of vitamin D to inhibit p38 through MKP5 is of interest to PCa prevention since p38 is activated by oxidative stress, hypoxia and inflammation, all of which contribute to PCa development (13-16). In particular, inflammation plays a causal role in the progression of many cancers including liver, bladder and gastric cancers (17) and a similar role for inflammation in the development of PCa is now emerging (16, 18, 19). The strongest evidence linking inflammation to PCa is from the recent findings that show regular administration of non-steroidal anti-inflammatory drugs (NSAIDS) significantly decreases PCa risk in older men by 60-80% (16, 20). Also, men with chronic and/or acute inflammation of the prostate, either in the form of prostatitis or a sexually transmitted disease, have an increased risk of developing PCa (15, 21).

Inhibitors of p38 are classically anti-inflammatory, suggesting that some of the activities attributed to vitamin D, including prostate cancer prevention, may be a result of p38 inhibition and decreased inflammation. One of the downstream consequences of p38 protein kinase pathway activation is an increase in pro-inflammatory cytokine production in order to amplify the inflammatory response (22, 23). Interleukin-6 (IL-6) is a p38-regulated pleiotropic cytokine that has been historically associated with PCa (24, 25). Elevated levels of IL-6 are found in the serum of PCa patients and primary PCa tumors over-express IL-6 (24, 26). IL-6 is also involved in the progression of PCa to androgen-independent PCa because it can facilitate androgen receptor signaling in the absence of androgens (25, 27, 28).

Since there is increasing evidence linking inflammation to PCa development, our studies focused on characterizing regulation of IL-6 production via p38 inhibition by 1,25D as a potentially significant cancer prevention activity. Using primary epithelial cell cultures (E-PZ) derived from the normal peripheral zone (the major site of origin of prostatic adenocarcinomas(29)) as our model system, we characterized MKP5 induction by 1,25D and revealed MKP5 as the mediator of p38 kinase inhibition and decreased IL-6 production by 1,25D in normal prostatic epithelial cells.

Materials and Methods

Cell Culture and Reagents. Human primary prostatic epithelial and stromal cells were derived from radical prostatectomy specimens. The patients did not have prior chemical, hormonal or radiation therapy. Histological characterization and culture of the prostate cells was as previously described (30). Epithelial cells (E-PZ) were cultured in supplemented MCDB 105 (Sigma-Aldrich, St. Louis, MO) or PFMR-4A as previously described (30). Stromal cells (F-PZ) were cultured in MCDB 105/10 % fetal bovine serum (FBS). pRNS1-1 cells are immortalized E-PZ cells (31) and are cultured in KSFM (Invitrogen, Carlsbad, CA). Human immortalized PCa cell lines LNCaP, PC-3 and DU 145 were acquired from ATCC (Manassas, VA). LNCaP cells were cultured in MCDB 105/10 % FBS, and PC-3 and DU 145 in DMEM (Invitrogen)/10 % FBS. Keratinocytes were obtained from Cambrex (East Rutherford, NJ) and cultured according to the supplier's instructions. All chemicals were obtained from Sigma-Aldrich unless otherwise noted. 1,25-dihydroxyvitamin D_3 (Biomol International, Plymouth Meeting, PA) was reconstituted in 100% ethanol at 10 mM and stored at -20ºC.

RNA isolation and quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). RNA was isolated from cells using Trizol™ (Invitrogen). Briefly, cells were lysed in Trizol followed by chloroform extraction. The aqueous phase was precipitated in 100% isopropanol and the pellet was further washed in 75% ethanol before resuspension in water. RNA concentration and quality were determined by absorbance ratio at 260/280 nm using a UV spectrophotometer. Total RNA (2 μg) was reverse transcribed using Thermoscript RT (Invitrogen). Resulting cDNA was used for qPCR amplification with gene specific primers and the DyNAMO Sybr Green kit (Finnzymes, Espoo, Finland) in the Opticon 2 thermocycler (MJ Research, South San Francisco, CA). PCR conditions for all primer sets were optimized and have similar amplification efficiency under the following conditions: 95ºC 5', 34X (95ºC 30', 58ºC 30', 72ºC 30'), 72ºC 5', melting curve 65º-95ºC. Relative mRNA levels were calculated

from the point where each curve crossed the threshold line (Ct) using the equation: Rel. Value = $2^{-[Ct(control) - Ct(test)]test\ gene} / 2^{-[Ct(control) - Ct(test)]housekeeping\ gene}$ (32). Reactions were performed in triplicate and the values normalized to the expression of the housekeeping gene TATA-box binding protein (TBP) (33). Primer sets were TBP: 5'-tgctgagaagagtgtgctggag-3' and 5'-tctgaataggctgtggggtc-3'; total MKP5: 5'atcttgcccttcctgttcct-3' and 5'-attggtcgtttgcctttgac-3'; MKP5 isoform 1-specific: 5'-tgaatgtgcgagtccatagc-3' and 5'-gttagcagggcaggtggtag-3'; MKP5 isoform 2-specific: 5'-tggatgcagctgagattctg-3' and 5'- ggttctgcttgttgctgtca-3'; MKP5 isoform 3 - specific: 5'-atttatgaagtggacttagt-3' and 5'- ggttctgcttgttgctgtca-3'; CYP24: 5'-ggcaacagttctgggtgaat-3' and 5'- tatttgcggacaatccaaca-3'.

Cell lysate preparation and immunoblot. Cells were lysed in ice-cold 1X Cell Lysis Buffer (Cell Signaling, Beverly, MA) containing 1 μmol/L phenylmethylsulfonyl fluoride (PMSF) and 100 nmol/L okadaic acid. Cells were briefly disrupted by sonication and insoluble cell debris removed by centrifugation at 15,000 x g, 4º C. Protein concentrations of the cell lysates were quantified using the BioRad Protein Dye (BioRad, Hercules, CA). Cell lysates were used fresh or stored at -70ºC. Cell lysates (10-30 μg) were mixed with LDS NuPAGE Sample Buffer and separated by electrophoresis through 10% NuPAGE Bis-Tris Gels (Invitrogen) and transferred onto PVDF membrane. Fresh cell lysates were used for analysis of phosphorylated proteins. Membranes were probed with the following primary antibodies: anti-phospho-p38 rabbit polyclonal and anti-p38 rabbit polyclonal from Cell Signaling, anti-vitamin D receptor (VDR) monoclonal (Santa Cruz Biotech, Santa Cruz, CA), and monoclonal anti-actin (Santa Cruz Biotech). Following primary antibody incubation overnight at 4ºC, the blots were incubated with appropriate secondary horseradish peroxidase conjugated-antibodies (Cell Signaling) and developed with HyGlo ECL reagent (Denville Scientific, Metuchen, NJ).

siRNA transfection. Cells at 75% confluency were transfected with 10 nmol/L of negative control (Ambion, Austin, TX), VDR-specific (Santa Cruz Biotech) or MKP5-specific

siRNA (Ambion) using siPORT NeoFX (Ambion). Cells were used for experiments after transfection as indicated in results and figure legends.

Chromatin immunoprecipitation (ChIP). ChIP assays were carried out using Upstate Biologics (Waltham, MA) protocol and reagents. Briefly, cells (1-100 mm dish per treatment) were cross-linked with 1% formaldehyde, harvested and sonicated prior to immunoprecipitation. One μg each of anti-VDR (H-81) and anti-VDR (N-20) rabbit polyclonal antibodies (Santa Cruz Biotech) were used for overnight 4ºC immunoprecipitation. Protein A-agarose beads were then used to pull down immune complexes. Beads were washed then reverse cross-linked with NaCl at 65ºC. The DNA was extracted with spin columns. PCR (30 cycles) was performed on 10% of the recovered DNA using primers flanking the vitamin D response element (VDRE) in the MKP5 promoter; VDRE-MKP5: 5'-ccagagccgagtgcaaatag-3' and 5'-gcaactttcctgcagttcc-3'. Primers for the GAPDH-promoter (5'-cggctactagcggttttacg-3' and 5'-aagaagatgcggctgactgt-3') were used as a negative control. PCR products were electrophoresed through 1.5% agarose gels containing 1 μg/ml of ethidium bromide and visualized by UV.

Ultraviolet (UV) irradiation. Prostate cell cultures were exposed to 1000 J/m^2 of UVB irradiation using calibrated UVB bulbs with a Kodaceal filter. The lid of the cell culture dish and phenol-red containing media were both removed during UV irradiation.

In vitro p38 kinase assay. All reagents, antibodies and protocol for this assay were supplied by Cell Signaling Technologies. Fresh cell lysate was prepared as described above under *Cell lysate preparation and immunoblot.* Cell lysates containing 250 μg of protein were incubated overnight with immobilized phospho-p38 monoclonal antibody to immunprecipitate activated p38. Bead-immune complexes were washed and resuspended in 1X Kinase Buffer containing 200 μmol/L adenosine tri-phosphate (ATP) and recombinant ATF-2 fusion protein as the substrate and incubated at 30ºC for 30 minutes to allow phosphorylation of ATF-2. Reactions were terminated by addition of LDS sample buffer. Samples were then heated at

95ºC for 5 minutes and kinase activity was determined by immunoblot analysis with phospho-ATF-2 antibody. Input protein (20 μg) was also immunobloted and probed with anti-p38.

IL-6 ELISA. Prostate cells (10^5) were plated in 24-well culture plates. After 24 hours fresh media containing various agents were added (as described in results and figure legends). Conditioned media were collected following treatment and used at a 2:1 dilution to determine amount of secreted IL-6 with the Human IL-6 ELISA Kit II (BD Biosciences, San Diego, CA). Results were calculated from a standard curve and are expressed as pg/ml IL-6 per 10^6 cells or pg/10^5 cells.

IL-6 promoter activity. A pGL3 luciferase construct containing a 651 bp fragment of the IL-6 promoter and pRL-null-renilla (Promega, Madison, WI) were transiently transfected into E-PZ cells using NeoFX reagent. pGL3-IL6 was a generous gift from Dr. Oliver Eikelberg at the University of Giessen, Germany. Eight hours after transfection, cells were treated with vehicle or 50 nmol/L 1,25D. Cells were UV irradiated 14 hours after vehicle or 1,25D treatment. Luciferase activity was measured 24 hours after UV using the Dual-luciferase Assay Kit (Promega). The ratio of luciferase to renilla-luciferase was determined to correct for transfection efficiency.

RESULTS

1,25D increases MKP5 mRNA expression in primary cultures of normal prostatic epithelial cells. We previously showed that MKP5 mRNA was increased 3- to 10-fold after 6 hours of treatment with 50 nmol/L of 1,25D in three independent primary cultures of normal human prostatic epithelial cells (E-PZ) (10). The optimal concentration of 1,25D required to upregulate MKP5 mRNA was determined by qRT-PCR. A dose-response curve showed that 1 nmol/L of 1,25D was sufficient to increase MKP5 mRNA in E-PZ cells by 6 hours, but 50 nmol/L of 1,25D was required to maintain MKP5 mRNA upregulation at 24 hours (Fig. 1*A*). Higher concentrations of 1,25D are needed at the time points greater than 12 hours because high density E-PZ cells rapidly metabolize and inactivate 1,25D (34). In all subsequent experiments, 50 nmol/L of 1,25D was used to treat subconfluent cultures of E-PZ cells. Upon treatment with 50 nmol/L of 1,25D, increased MKP5 expression was observed as early as 3 hours and maintained for 24 hours in E-PZ cells (Fig. 1*B*).

The MKP5 gene is located on chromosome 1 and is transcribed into three distinct mRNA splice-variants that putatively encode two different proteins (35). MKP5 splice-variant 1 encodes the full-length 52 kDa protein, while variants 2 and 3 both encode a truncated 16 kDa protein that only contains the dual specificity phosphatase domain (Fig. 1*C*). Since the biological significance of the MKP5 splice-variants has not yet been characterized, we examined the ability of 1,25D to regulate the mRNA expression of the three MKP5 splice -variants by qRT-PCR. All of the MKP5 mRNA splice-variants were induced following 6 hours of treatment with 50 nmol/L of 1,25D, but splice-variant 1 appeared to be most abundant and achieved the highest level of mRNA induction in the E-PZ cells (Fig. 1*C*). Primers within exons 3-5 of MKP5, the conserved region present in all MKP5 mRNAs, were used for all subsequent experiments.

Upregulation of MKP5 mRNA by 1,25D is VDR dependent and MKP5 promoter contains a putative VDRE that associates with VDR upon 1,25D treatment. The rapid induction of MKP5 mRNA by 1,25D suggested that MKP5 is a direct target of 1,25D. Direct

targets of 1,25D contain one or more vitamin D response elements (VDREs) in the promoter region which mediate transcriptional regulation by vitamin D receptor (VDR) binding. Knockdown of VDR levels by VDR-siRNA in E-PZ cells abolished the induction of MKP5 by 1,25D (Fig.2*A*), demonstrating that induction of MKP5 by 1,25D is VDR-dependent. The mRNA expression of vitamin D 24-hydroxylase (CYP24), a well characterized target of 1,25D, was similarly suppressed in cells transfected with VDR-siRNA whereas the expression of TATA-box binding protein (TBP), a housekeeping gene, was not affected by VDR-siRNA transfection. Upon sequence analysis, a putative VDRE was identified -1320 base pairs upstream of the 5' untranslated region in the MKP5 promoter (Fig. 2*B*). The putative MKP5-VDRE was highly similar to the characterized VDREs present in the promoters of CYP24 and parathyroid hormone-related protein (PTHrP). ChIP assay showed an increased interaction between VDR and the putative VDRE that exhibited time-dependent changes upon stimulation with 50 nmol/L of 1,25D in E-PZ cells (Fig. 2*C*). The cyclic nature of VDR interaction with the MKP5 promoter observed in the ChIP experiments is consistent with previously described interactions of nuclear hormone receptors with DNA (36). The siRNA and ChIP data together provide strong support that MKP5 is directly regulated by 1,25D at the transcription level.

MKP5 mediated p38 inactivation by 1,25D occurs in E-PZ cells and not in PCa cell lines. Phosphorylation of p38 is required for activation of p38 kinase activity. MKP5 dephosphorylates p38 thus reducing p38 kinase activity. Vitamin D has been shown to inhibit osmotic stress-stimulated p38 phosphorylation in keratinocytes (37). We observed a similar inhibition of phosphorylation of p38 by 1,25D in E-PZ cells (Fig. 3*A*). E-PZ cells were pre-treated with 1,25D for 14 hours in order to allow for sufficient upregulation of MKP5 protein Protein levels could not be directly monitored in this study due to lack of an appropriate antibody. After pre-treatment with 1,25D, osmotic stress-stimulated phosphorylated p38 levels were appreciably decreased compared to levels in cells not treated with 1,25D. Transfection with MKP5-specific siRNA attenuated induction of MKP5 mRNA by 1,25D compared to negative

control-siRNA (Fig. 3*B*) and abolished the suppression of p38 phosphorylation by 1,25D (Fig. 3*A*), implicating the induction of MKP5 in the inactivation of p38 by 1,25D.

We suspect that MKP5 upregulation by 1,25D is not unique to the prostatic epithelium. Since inactivation of p38 by 1,25D was reported in keratinocytes, we examined the expression of MKP5 in these cells. Similarly to prostatic epithelial cells, keratinocytes showed upregulation of MKP5 mRNA upon treatment with 1,25D, suggesting that MKP5 may mediate p38 inactivation in keratinocytes as well (Supplemental Fig. 1).

We examined the effect of 1,25D on MKP5 in various other prostate-derived cells in comparison to the primary cultures of E-PZ cells. The results showed that, like E-PZ cells, pRNS-1-1 cells also induce MKP5 (Fig. 3*C*). In contrast, prostate stromal cells (F-PZ) and established PCa cell lines (PC-3, LNCaP, DU 145) did not upregulate MKP5 mRNA following 1,25D treatment (Fig. 3*C*). pRNS-1-1 cells were generated by SV40 transformation and immortalization of E-PZ cells and are not growth inhibited by 1,25D, although they retain VDR and other responses to 1,25D (38). Prostatic stromal cells, too, express VDR and show certain responses to 1,25D despite lack of induction of MKP-5 in these cells by 1,25D. The prostate cancer cell lines PC-3, LNCaP and DU 145 all express VDR and respond to 1,25D in other ways (9). Immunoblot analysis showed that in DU145, PC-3, and LNCaP cells, 1,25D pre-treatment did not alter NaCl-induced p38 phosphorylation (Fig. 3*D*). These data suggest that MKP5 may specifically mediate 1,25D activity in normal prostate cells and that this activity is lost in PCa.

MKP5 mediates decreased IL-6 production in E-PZ cells by 1,25D. Published studies have shown that 1,25D inhibits UV-induced IL-6 production in keratinocytes, however, no mechanism has been proposed (39). IL-6 overexpression has been strongly associated with PCa progression and therefore inhibition of IL-6 may play an important role in PCa prevention. Since IL-6 induction is downstream of p38 activation and often dependent upon p38 activation

(23, 40), we tested the role of MKP5 in regulating IL-6 expression in E-PZ cells. An *in vitro* p38 kinase activity assay, using ATF-2 as the substrate, showed that 1,25D pre-treatment decreased basal and UV-induced p38 activity in E-PZ cells (Fig. 4*A*). The 1000 J/m^2 dose of UVB irradiation used in these experiments did not induce apoptosis or necrosis in E-PZ cells (data not shown). Consistent with p38 inactivation, secreted levels of IL-6 protein following UV treatment were suppressed by 1,25D pre-treatment (Fig. 4*B*). SB202190, a specific p38 inhibitor, similarly decreased UV-stimulated IL-6 production (Fig. 4*B*). Inhibition of IL-6 by 1,25D occurred at the mRNA level and this decrease was blocked by transient transfection with MKP5-siRNA (Fig. 4*C*). The primary mechanism for increased IL-6 production following p38 activation is through IL-6 mRNA stabilization rather than increased mRNA transcription (41). To determine if this is also true for 1,25D regulation of IL-6 mRNA, we examined the effect of 1,25D on IL-6 promoter activity in the absence or presence of UV irradiation. We found that neither UV irradiation nor 1,25D treatment significantly altered IL-6 promoter activity as determined by luciferase assay in E-PZ cells (Fig. 4*D1*), suggesting that 1,25D is not altering mRNA transcription. When 1 μmol/L of actinomycin D was used to inhibit new mRNA transcription, we observed that UV irradiation caused IL-6 mRNA stabilization and 1,25D pre-treatment decreased the UV-induced IL-6 mRNA stabilization (Fig. 4*D2*). Under basal conditions IL-6 mRNA half-life was less than 45 minutes in E-PZ cells. In UV-irradiated cells, IL-6 mRNA half-life increased to greater than 90 minutes, whereas UV-irradiation did not significantly alter IL-6 mRNA half-life in 1,25D pre-treated cells (Fig. 4*D2*).

1,25D inhibits TNFα-stimulated p38 activation and IL-6 production. The ability of 1,25D to inhibit p38 phosphorylation was further investigated using a more physiological stress, tumor necrosis factor (TNF)-α. TNFα is a pro-inflammatory cytokine released by inflammatory cells that can trigger cell proliferation, necrosis, apoptosis and induction of other cytokines (42). Interestingly, elevated serum levels of TNFα are associated with aggressive pathology and

decreased survival of PCa patients (26). In E-PZ cells, TNFα does not induce apoptosis but does significantly slow cell growth (43). TNFα binds cell surface receptors which signal through multiple pathways including p38 kinase to active gene expression of IL-6 and other cytokines (22). Immunoblot analysis showed that 50 ng/ml TNFα-stimulated p38 phosphorylation was attenuated by 1,25D pre-treatment, similar to the effect of 1,25D on UV-stimulated p38 phosphorylation in E-PZ cells (Fig. 5*A*). Changes in IL-6 mRNA and secreted protein levels were followed over a time course after TNFα stimulation and showed that 1,25D pre-treatment attenuated the initial production of IL-6 and completely inhibited the accumulation of IL-6 in the media (Fig. 5*B,C*).

Discussion

The purpose of these studies was to explore the potential significance of MKP5 in mediating PCa prevention by vitamin D. We had previously identified MKP5 as a target of 1,25D in normal human prostatic epithelial cells by microarray analysis (10). The results of our studies suggest that the ability of vitamin D to inhibit p38 signaling, via MKP5 upregulation, may be a significant anti-tumor activity of vitamin D (Fig. 6).

MKP5 is likely a direct target of 1,25D, regulated by a positive VDRE in the promoter region of the gene. 1,25D treatment of E-PZ cells produced a time- and dose-dependent increase in MKP5 mRNA that was dependent upon VDR expression. Furthermore, VDR was found to associate with the VDRE in the MKP5 promoter upon 1,25D treatment. These data support direct transcriptional activation of the MKP5 gene by 1,25D. Consistent with increased levels of MKP5, 1,25D inhibited p38 phosphorylation. MKP5-siRNA blocked p38 inactivation by 1,25D, which further demonstrated that MKP5 mediates p38 inactivation by 1,25D.

The ability of 1,25D to inactivate p38 led us to examine the regulation of IL-6, which is downstream of p38 activation (40), by 1,25D. Suppression of UV-stimulated IL-6 production by 1,25D was previously demonstrated in keratinocytes (39) and we suspected that similar activity could be mediated by MKP5 in normal prostatic epithelial cells. Using MKP5-siRNA, we demonstrated that 1,25D inhibited UV-stimulated p38 activity and IL-6 production in a MKP5-dependent manner in E-PZ cells. UV irradiation did not increase IL-6 promoter activity, but did increase mRNA half-life, indicating post-transcriptional regulation of IL-6 mRNA expression. 1,25D pre-treatment was able to attenuate the UV-stimulated increase in IL-6 mRNA half-life. Induction of IL-6 by a more physiologically relevant stress, TNFα, was similarly inhibited by 1,25D.

IL-6, as well as other interleukins (IL-1, IL-4, IL-8, IL-10, IL-11, IL-17), and/or their receptors are often overexpressed in PCa tissue and/or serum of PCa patients (25, 44-49). Elevated serum levels of IL-6 and TNFα are associated with aggressive pathology and

decreased survival of PCa patients (26). Increased IL-6 staining is observed in malignant prostate tissue compared to adjacent normal tissue and IL-6 also contributes to the development of hormone-refractory cancer by androgen-independent activation of the androgen receptor (50). Although pro-inflammatory factors are overexpressed in PCa, it is unclear whether increased levels of these factors are required for development of the cancer originally or are a consequence of the cancer. On one hand, inflammation may trigger the initial expression of these factors in normal prostate tissue and when PCa arises, the PCa cells maintain these features for a survival advantage. On the other hand, since there is significant inflammatory infiltrate in PCa lesions, the possibility of the inflammatory genes becoming expressed later in PCa development cannot be excluded. If the former situation occurs, then our results suggest that the ability of 1,25D to suppress the synthesis of IL-6, and perhaps other inflammatory factors, may be a key component in blocking carcinogenic events associated with inflammation.

Another important finding from our study was that 1,25D upregulated MKP5 only in the cells derived from normal prostatic epithelium, including pRNS-1-1 cells. Although pRNS-1-1 cells originated from normal prostatic epithelial cells (E-PZ), they were immortalized by SV40 TAg and are no longer sensitive to growth-inhibition by 1,25D (38, 51). Yet, pRNS-1-1 cells retain functional VDRs and upregulate vitamin D 24-hydroxylase, a classic vitamin D target gene, in response to 1,25D. Stromal cells (F-PZ) derived from normal prostatic tissue did not induce MKP5 when treated with 1,25D, although these cells express VDR and are growth-inhibited by 1,25D. PCa cell lines also are growth inhibited by 1,25D (9) while MKP5 was not upregulated in these cell lines. Together these observations demonstrate that 1) induction of MKP5 by 1,25D is specific to normal prostatic epithelial cells and does not occur in normal prostatic stromal cells, 2) induction of MKP5 is independent of growth inhibition by 1,25D, and 3) lack of 1,25D-induced MKP5 in PCa cell lines is not a result of immortalization per se since immortalized pRNS-1-1 cells upregulate MKP5 in response to 1,25D. From the qRT-PCR data,

it also appears that the basal levels of MKP5 are lower in the PCa cell lines compared to normal prostatic epithelial cells. Since the established PCa cell lines have low levels of MKP5 and are unable to induce MKP5 in response to 1,25D, it is tempting to speculate that loss of MKP5 may occur in PCa as the result of selective pressure to eliminate tumor suppressor activity of MKP5 and/or 1,25D. A number of MKP family members have been suggested to be tumor-suppressors. In PCa MKP1 has been shown to be downregulated (52). Candidate MKP tumor suppressors in other malignancies include MKP7, which is frequently deleted in lymphoblastic leukemia (53), MKP3, hypermethylated or deleted in pancreatic cancer (54, 55), and MKP2, which is deleted in breast carcinoma (56).

The link between inhibition of p38 by 1,25D via MKP5 and PCa prevention becomes more apparent when the many different ways the p38 pathway may become activated are considered. In addition to inflammatory cytokines, osmotic stress and UV irradiation (which we used in these studies), reactive oxygen species (ROS) and hypoxia also activate p38 (57). ROS can amplify p38 activation because they are generated during hypoxia and as a by product of inflammation (14, 17) (Fig. 6). Hypoxia has been implicated in PCa metastasis and progression to androgen independence (14).

In addition to a potential role in PCa prevention, p38 inhibition by 1,25D via MKP5 may be farther reaching and mitigate activities of vitamin D in other tissues. Recent cDNA microarray analyses have shown MKP5 upregulation by vitamin D in skin (58) and colon (59) cells, although biological studies have yet not been done in these tissues. Furthermore, the overall immunomodulatory activity of vitamin D on the VDR-expressing cells of the innate and adaptive immune system (60) is highly similar to the immunomodulatory role of MKP5 that was shown by Zhang et al. using MKP5 knockout mice (61). The mechanism by which vitamin D reduced IL-6 mRNA stability, through MKP5-mediated p38 inactivation, may also be responsible for down-regulation of other mRNAs. Activation of the p38 pathway causes a robust and rapid increase in inflammatory response proteins by mRNA stabilization and increased translation

through AU-rich elements in the 3'-untranslated region (3'-UTR) (62). Post-transcriptional regulation of inflammatory genes is the basis for the anti-inflammatory activity of p38 inhibitors (62). AU-rich elements have been identified in several other genes down-regulated by vitamin D, including cyclooxygense-2 (COX-2), c-fos, urokinase plasminogen activator (uPA), TNFα and granulocyte-macrophage colony-stimulating factor (GM-CSF) (63-67). Decreased mRNA stabilization by vitamin D has been shown to mediate down-regulation of GM-CSF (67) and we hypothesize that this mechanism will have a role in decreasing the other AU-rich 3'-UTR-containing pro-inflammatory mRNAs as well by vitamin D.

It is becoming apparent that inflammation, both chronic and acute, contributes to PCa development. In a recent review article, Nelson et al. stated, "When considered together, evidence from prostate cancer epidemiology, genetics and molecular pathogenesis converge on the hypothesis that prostate inflammation and/or infection may be a cause of prostate cancer." (19). If inflammation is a significant risk factor for PCa, then PCa prevention will best be achieved with agents, like vitamin D, that inhibit inflammation and/or decrease the cellular stress response that accompanies inflammation. Our study shows that MKP5 is a mediator of anti-inflammatory effects of 1,25D and suggests that vitamin D may play a significant role in PCa prevention by facilitating p38 inhibition and reduced IL-6 production in normal human prostatic epithelial cells.

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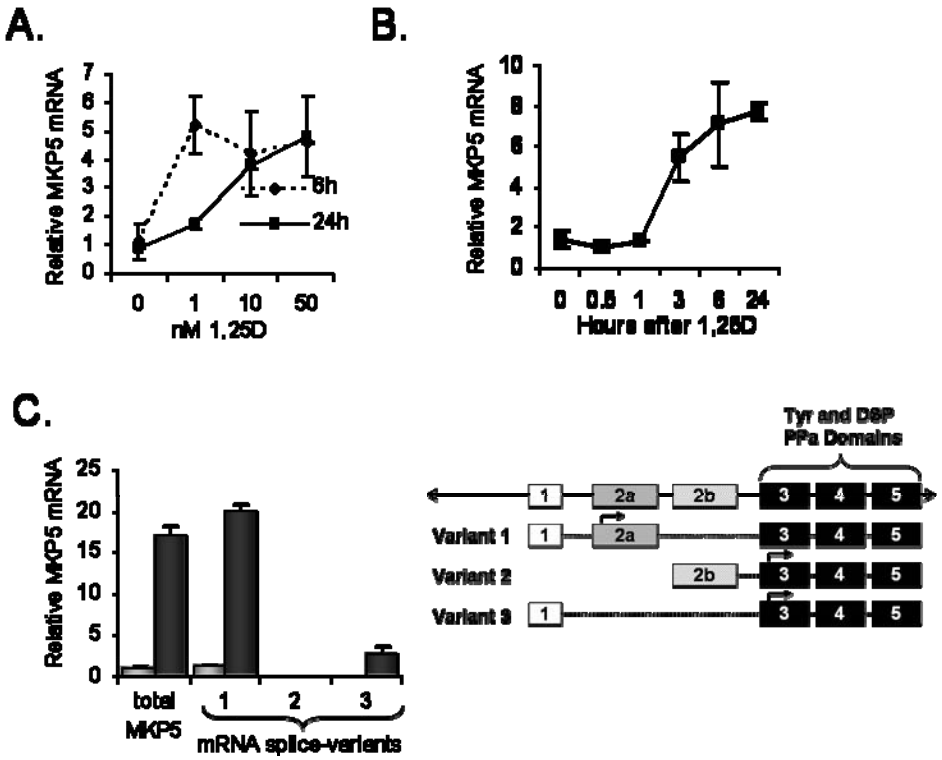


Figure 1. 1,25D increases MKP5 mRNA expression in primary cultures of normal prostatic epithelial cells (E-PZ). qRT-PCR measurement of MKP5 mRNA levels in E-PZ cells, **A**, 6 hours (dashed line) and 24 hours (solid line) after treatment with 1 nM, 10 nM and 50 nM 1,25D and, **B**, 0.5, 1, 3, 6 and 24 hours following 50 nM 1,25D treatment. **C**, qRT-PCR analysis of MKP5 mRNA splice variants in E-PZ cells after 6 hour vehicle (light bars) or 50 nM 1,25D (dark bars) treatment (mRNA levels for isoform 2 treated with vehicle and 1,25D are 0.003±0.0007 and 0.014±0.0004, respectively but are not visible on this graph). Diagram of MKP5 mRNA splice-variants. qRT-PCR results are shown relative to untreated control and normalized to expression of housekeeping gene TBP. Each experiment is run in triplicate and graphs are representative of 2 or more separate experiments with different patient-derived E-PZ cells. Error bars represent standard deviation.

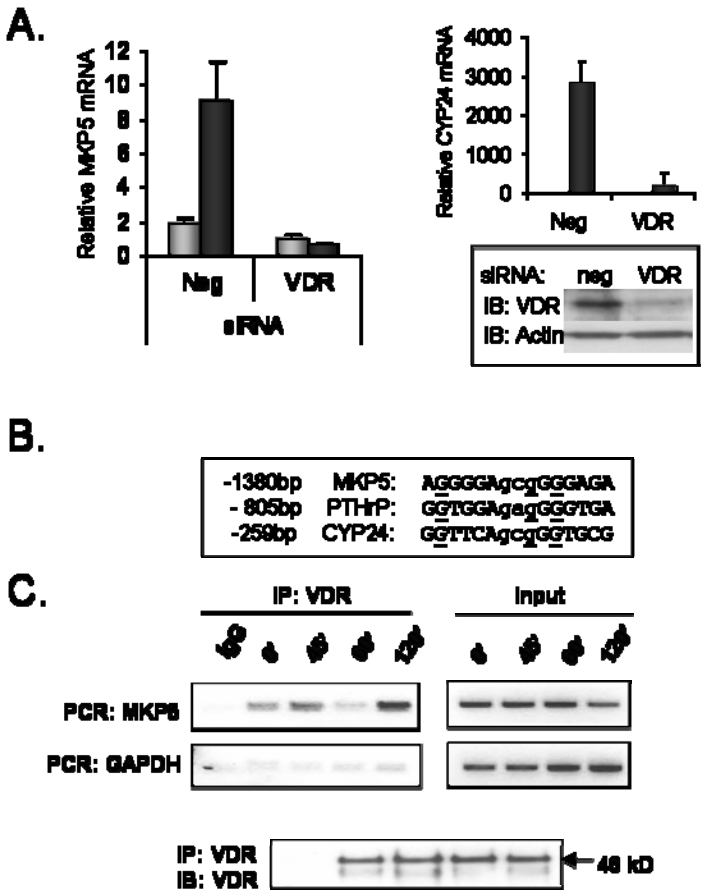


Figure 2. Upregulation of MKP5 mRNA by 1,25D is VDR dependent and MKP5 promoter contains a putative VDRE that associates with VDR upon 1,25D treatment. **A**, qRT-PCR analysis of MKP5 mRNA 6 hours after treatment with vehicle (light bars) or 50 nM 1,25D (dark bars) in E-PZ cells that were transfected for 24 hours with negative control siRNA or VDR-siRNA. CYP24 gene expression included as positive control for VDR knockdown (untreated mRNA level of CYP24 is equal to one and is not visible on this graph) and immunoblot of nuclear lysate (10 μg) verified VDR knockdown by the siRNA. **B**, Diagram of putative VDRE located -1380bp of 5'UTR in MKP5 promoter aligned with validated VDREs in PTHrP and CYP24. **C**, Chromatin immunoprecipitation analysis (ChIP) and PCR of putative-VDRE in MKP5 promoter and GAPDH promoter following 50 nM 1,25D treatment, using rabbit polyclonal VDR antibody for pulldown. Immunoblot of protein precipitate probed with mouse monoclonal

VDR antibody shows specific pulldown of VDR. Results are representative of 4 or more separate experiments with different patient-derived E-PZ cells. qRT-PCR results are shown relative to untreated control and normalized to expression of housekeeping gene TBP. Each experiment is run in triplicate and graphs are representative of 2 or more separate experiments. Error bars represent standard deviation.

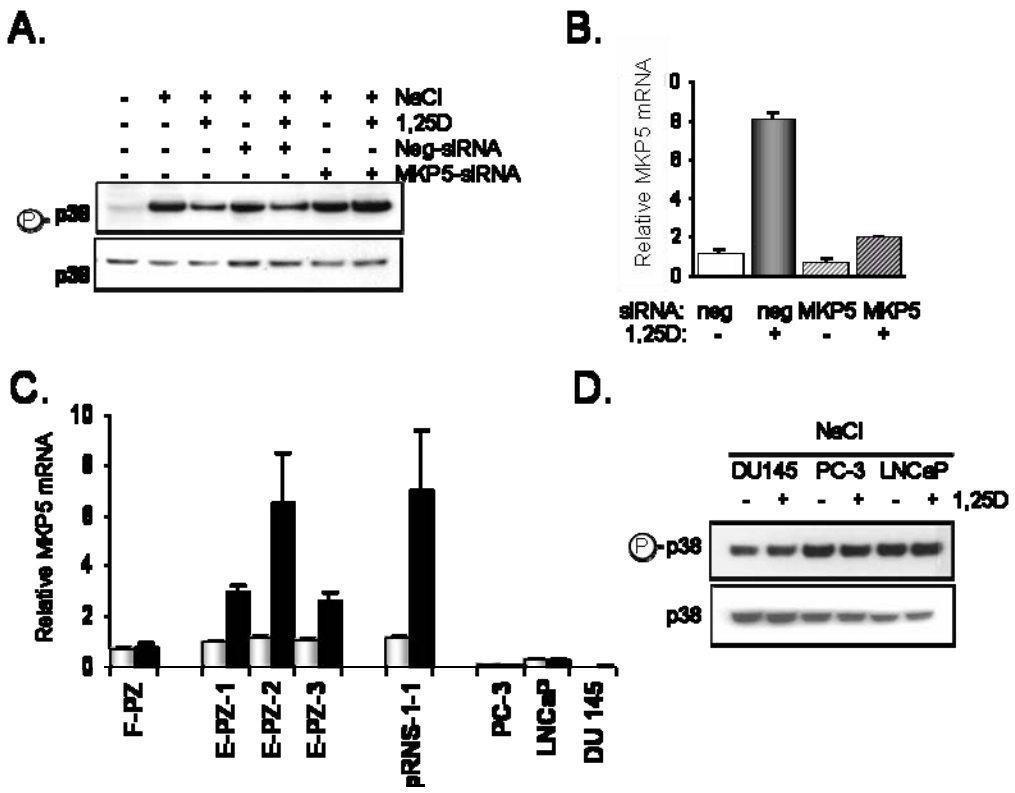


Figure 3. MKP5 mediated p38 inactivation by 1,25D occurs in E-PZ cells and not in PCa cell lines**. A**, immunoblot analysis of p38 phosphorylation in 20 μg of E-PZ cell lysate 20 minutes after treatment with 0.5 M NaCl. Prior to NaCl treatment, E-PZ cells were transfected with negative control or MKP5-siRNA for 4 hours then treated with vehicle or 50 nM 1,25D for 14 hours. **B**, qRT-PCR analysis of MKP5 mRNA expression in E-PZ cells transfected with negative control siRNA (open bars) or MKP5 siRNA (striped bars) 4 hours prior to vehicle (light bars) or 1,25D (shaded bars) treatment for 12 hours. Results are representative of at least 3 separate experiments with different patient-derived E-PZ cells. **C**, qRT-PCR analysis of MKP5 mRNA 6 hours after treatment with vehicle (light bars) or 50 nM 1,25D (dark bars) in primary cultures of prostate stroma (F-PZ), in three different cultures of normal primary prostatic epithelial cells (derived from normal peripheral zone designated E-PZ), in prostate cancer cell lines LNCaP, PC-3 and DU 145. qRT-PCR results are displayed relative to control and normalized to expression of the housekeeping gene TBP. Error bars represent standard deviation. **D**,

Immunoblot analysis of p38 phosphorylation in DU 145, PC-3 and LNCaP cells 20 minutes after 0.5 M NaCl pretreated for 14 hours with vehicle or 50 nM 1,25D. Results are displayed relative to negative siRNA control and normalized to expression of the housekeeping gene TBP. Error bars represent standard deviation or triplicate samples.

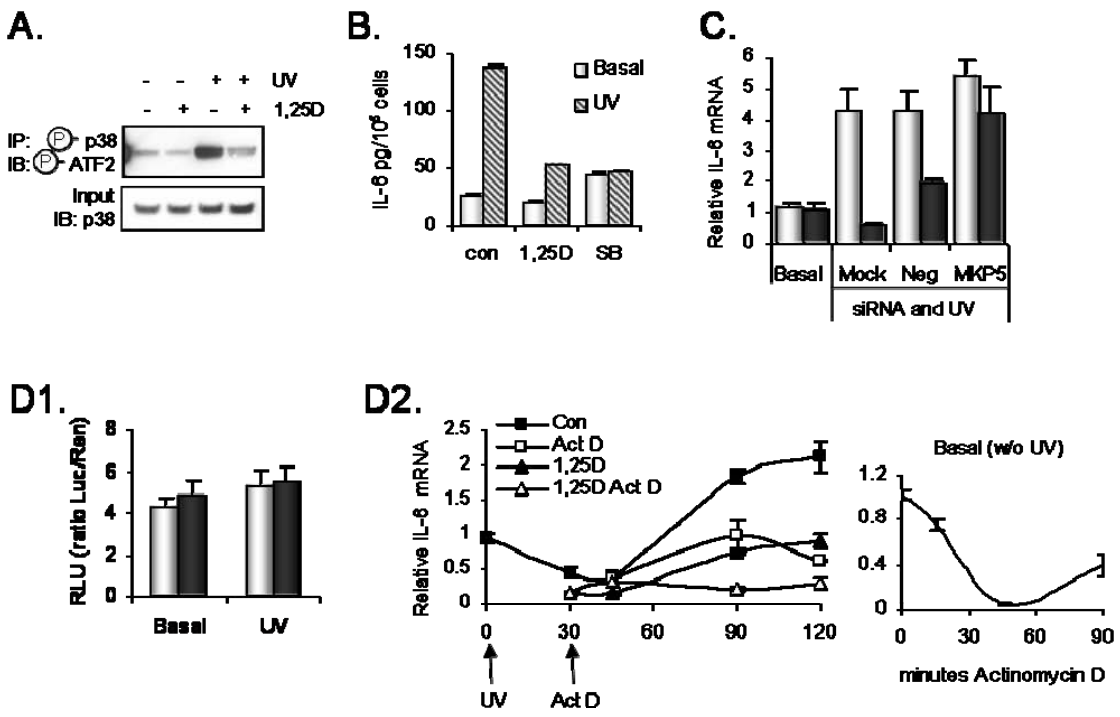


Figure 4. MKP5 mediates decreased IL-6 production in E-PZ cells by 1,25D. **A**, in vitro p38 kinase activity assay, using ATF2 as a substrate, in E-PZ cells 20 minutes after 1000 J/m^2 UVB irradiation in E-PZ cells pretreated 14 hours with vehicle or 50 nM 1,25D. **B**, ELISA measurement of secreted IL-6 in cell culture media 24 hours after UVB irradiation (hatched bars) in E-PZ cells pretreated with vehicle, 50 nM 1,25D for 14 hours or 1 μM SB202190 for 1 hour. **C**, qRT-PCR measurement of IL-6 mRNA in E-PZ cells 24 hours after UV irradiation. Cells were either transiently transfected with neg-siRNA or MKP5-siRNA for 4 hours then pretreated for 14 hours with either vehicle (light bars) or 50 nM 1,25D (dark bars) before UV. **D1**, luciferase activity of pGL3-IL6 24 hours after UV irradiation in E-PZ cells pretreated for 14 hours with vehicle (light bars) or 50 nM 1,25D (dark bars). **D2**, qRT-PCR analysis of IL-6 mRNA levels in E-PZ cells at 30 to 120 minutes following UV irradiation. Cells were treated with vehicle (black symbols) or 1 μM actinomycin D (open symbols) 30 minutes after UV and pretreated 14 hours prior to UV with vehicle (squares) or 50 nM 1,25D (triangles). IL-6 mRNA

levels under basal conditions after 15 to 90 minutes of 1 μM actinomycin D treatment shown as control.

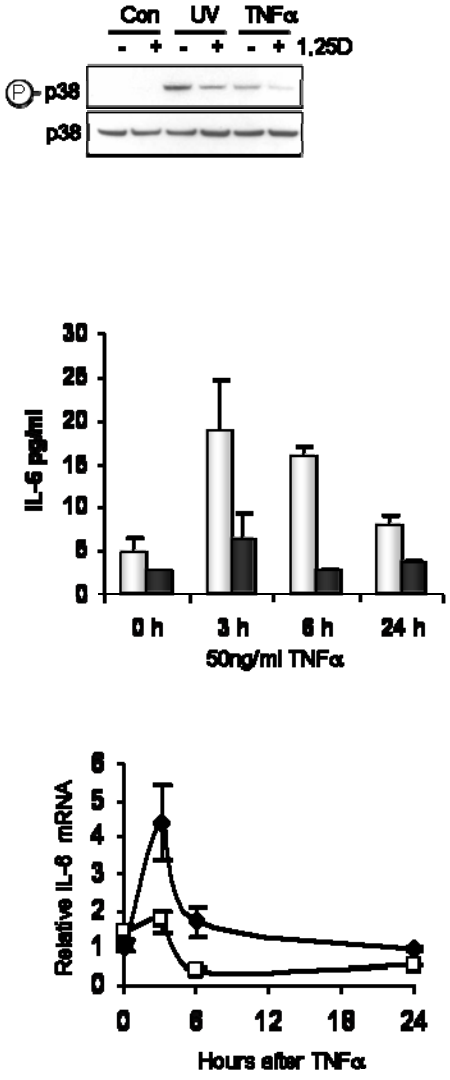


Figure 5. 1,25D inhibits TNFα-stimulated p38 phosphorylation and IL-6 production. **A**, immunoblot analysis of p38 phosphorylation 20 minutes after treatment with 1000 J/m^2 UVB or 50 ng/ml TNFα in E-PZ cells pretreated 14 hours with vehicle or 50 nM 1,25D. **B**, ELISA measurement of secreted IL-6 in cell culture media 3, 6 and 24 hours after 50 ng/ml TNFα in E-PZ cells pretreated 14 hours with vehicle (light bars) or 50 nM 1,25D (dark bars). **C**, qRT-PCR

analysis of IL-6 mRNA 3, 6 and 24 hours after 50 ng/ml TNFα in E-PZ cells pretreated 24 hours with vehicle (black diamonds) or 50 nM 1,25D (open squares). IL-6 gene expression relative to untreated control and normalized to expression of the housekeeping gene TBP. Error bars represent standard deviation of triplicate sample.

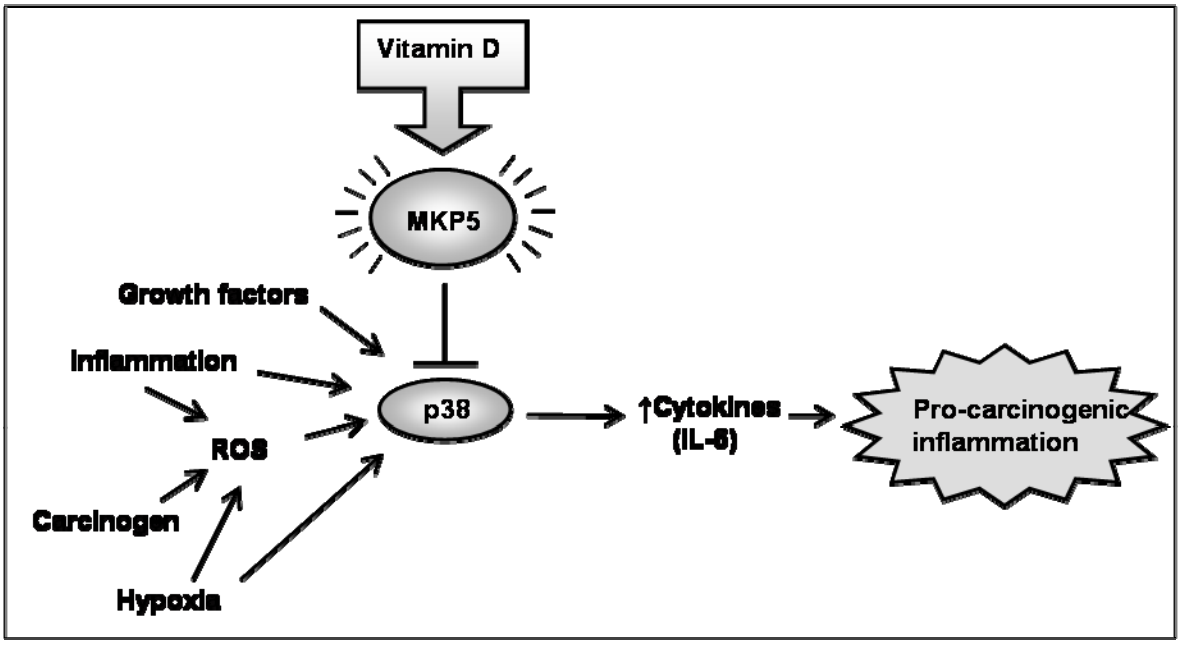
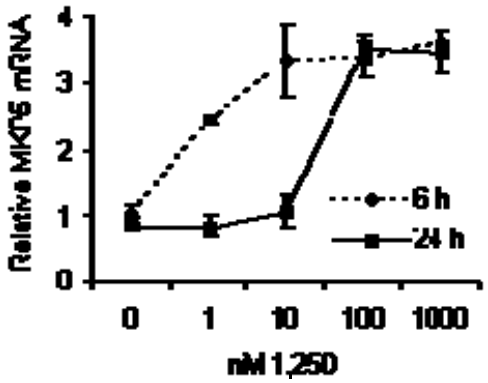


Figure 6. Proposed mechanism for anti-inflammatory and prostate cancer prevention by vitamin D.



Supplemental Figure S1. qRT-PCR analysis of MKP5 mRNA in primary normal human adult keratinocytes treated with vehicle, 1, 10, 100 and 1000 nM 1,25D for 6 (dashed line) and 24 hours (solid line). MKP5 mRNA expression measured by qRT-PCR and reported relative to vehicle control and normalized to expression of housekeeping gene TBP. Error bars represent standard deviation.

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