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Activation of Rapid Signaling Pathways Does Not Contribute to 1 α ,25-Dihydroxyvitamin D₃-Induced Growth Inhibition of Mouse Prostate Epithelial Progenitor Cells

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Abstract

The active form of vitamin D, 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D) inhibits the growth of prostate epithelial cells, however the underlying mechanisms have not been clearly delineated. In the current study, the impact of 1,25(OH)₂D on the rapid activation of extracellular-regulated kinase (ERK) 1/2 and protein kinase C α (PKC α), and the role of these pathways in growth inhibition was examined in immortalized mouse prostate epithelial cells, MPEC3, that exhibit stem/progenitor cell characteristics. 1,25(OH)₂D treatment suppressed the growth of MPEC3 in a dose and time dependent manner (e.g., 21% reduction at three days with 100 nM 1,25(OH)₂D treatment). However, ERK1/2 activity was not altered by 100 nM 1,25(OH)₂D treatment for time points from 1 min to 1 h in either serum-containing or serum-free medium. Similarly, PKC α activation (translocation onto the plasma membrane) was not regulated by short-term treatment of 100 nM 1,25(OH)₂D. In conclusion, 1,25(OH)₂D did not mediate rapid activation of ERK1/2 or PKC α in MPEC3 and therefore the growth inhibitory effect of 1,25(OH)₂D is independent of rapid activation of these signaling pathways in this cell type.

Keywords

PROSTATE; VITAMIN D; 1,25-DIHYDROXYVITAMIN D; MAPK; PROTEIN KINASE C; GROWTH INHIBITION

The active hormonal form of vitamin D, 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D), has been proposed to mediate protection from prostate cancer [Schwartz and Hulka, 1990; Skowronski et al., 1993]. The mechanism of 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D) cellular actions involves both traditional, genomic signaling pathway through the nuclear vitamin D receptor (nVDR) and rapid (within minutes or seconds) activation of kinases. The nVDR plays a critical role in mediating ligand-induced transcriptional regulation of a variety of genes that are important for cell proliferation, differentiation, and apoptosis. In addition, several kinases have been shown to be rapidly regulated by 1,25(OH)₂D, including extracellular-regulated kinase (ERK) 1/2 [Beno et al., 1995; Marcinkowska et al., 1997; Song et al., 1998; Chen et al., 1999; Morelli et al., 2001; Buitrago et al., 2001a,b; Galbiati et al., 2002; Schwartz et al., 2002; Johansen et al., 2003; Capiati et al., 2004; Pardo and de Boland, 2004] and protein kinase C α (PKC α) [Balogh et al., 2000; Capiati et al., 2000; Rivera-Bermudez et al., 2002; Boyan et al., 2003; Schwartz et al., 2003; Wali et al., 2003], which may contribute to the regulation of

genes that are not direct targets of nVDR [Farach-Carson and Davis, 2003]. However, the mechanisms mediating growth inhibition by vitamin D that lead to prostate cancer prevention, as well as therapy by vitamin D analogues, are not known definitively. One hypothesis is that these effects are not mediated by transcriptional activation requiring the VDR but by the rapid, membrane-initiated actions of $1,25(\text{OH})_2\text{D}$.

The regulation of ERK1/2 by $1,25(\text{OH})_2\text{D}$ has been shown in a variety of cell types. ERK1/2 is a member of the mitogen-activated protein kinases (MAPK). Upon upstream activation by extracellular stimuli, such as growth factors, ERK1/2 phosphorylates a variety of downstream proteins including transcription factors, and therefore modulates their activity and corresponding gene transcription. Rapid activation of ERK1/2 by $1,25(\text{OH})_2\text{D}$ has been observed in muscle cells [Buitrago et al., 2001b; Morelli et al., 2001], enterocytes [Pardo and de Boland, 2004], costochondral chondrocytes [Schwartz et al., 2002], colon carcinoma [Chen et al., 1999], insulinoma cells [Galbiati et al., 2002], hepatic cells [Beno et al., 1995], acute promyelocytic leukemia cells [Marcinkowska et al., 1997; Song et al., 1998], and keratinocytes [Marcinkowska et al., 1997; Johansen et al., 2003].

Another rapid signal previously reported to be induced by $1,25(\text{OH})_2\text{D}$ is PKC α [Balogh et al., 2000; Capiati et al., 2000; Rivera-Bermudez et al., 2002; Boyan et al., 2003; Schwartz et al., 2003; Wali et al., 2003]. PKC α is classified as a classical PKCs (cPKC), and a downstream target of extracellular stimulated receptors, including G-protein coupled receptors (GPCRs) [Breitkreutz et al., 2007]. Activated GPCR induces phospholipase C (PLC) activity to hydrolyze 4,5-bisphosphate (PIP₂) and consequently produce inositol 1,4,5-trisphosphate (IP₃) into cytosol and diacylglycerol (DAG) on the plasma membrane [Breitkreutz et al., 2007]. IP₃ triggers calcium release through the IP₃ receptor located on endoplasmic reticulum. The interaction of both DAG and calcium with PKC α brings PKC α to the plasma membrane and activates its kinase activity [Breitkreutz et al., 2007]. Activated PKC α phosphorylates proteins on serine/threonine residues and thereby regulating their activity [Breitkreutz et al., 2007]. Rapid PKC activation by $1,25(\text{OH})_2\text{D}$ has been shown in myoblasts and myotubes [Capiati et al., 2000], duodenal mucosae [Balogh et al., 2000], chondrocytes [Boyan et al., 2003; Schwartz et al., 2003], osteoblasts [Wali et al., 2003], osteosarcoma cell line [Rivera-Bermudez et al., 2002].

Induction of cell cycle arrest by $1,25(\text{OH})_2\text{D}$ has been observed in both normal and transformed prostate epithelial cells [Miller et al., 1992; Skowronski et al., 1993, 1995; Peehl et al., 1994; Blutt et al., 1997; Zhuang and Burnstein, 1998; Sprenger et al., 2001; Rao et al., 2002]. However, the activation of ERK1/2 and PKC α by $1,25(\text{OH})_2\text{D}$, and the role of these pathways in $1,25(\text{OH})_2\text{D}$ -mediated growth inhibition have not been investigated in prostate progenitor/stem cells. The cancer stem cell theory proposes the origin of prostate cancer is from a small prostate stem cell population [Bonkhoff, 1996]. These cells have the self-renewal and multilineage differentiation properties of normal stem cells. They are responsible for tumor reoccurrence because of their resistance to radiotherapy and androgen-deprivation therapy [Maitland et al., 2006]. Therefore it is critical to study the impact of potential chemopreventive agents in untransformed progenitor cells. The purpose of these studies is to investigate whether $1,25(\text{OH})_2\text{D}$ rapidly activates ERK1/2 and PKC α and whether these rapid signaling pathways play a role in $1,25(\text{OH})_2\text{D}$ -mediated growth inhibition in normal prostate progenitor cells.

MATERIALS AND METHODS

MATERIALS

Unless otherwise noted, all chemicals were obtained from Sigma (St. Louis, MO). Bicinchoninic acid (BCA) assay was obtained from Pierce (Rockford, IL), $1,25(\text{OH})_2\text{D}$ was from Biomol (Plymouth Meeting, PA) and Lipofectamine 2000 from Invitrogen (Carlsbad,

CA). The vector expressing a fusion of PKC α and enhanced green fluorescent protein (PKC α -EGFP) and the GFP monoclonal antibody were from Clontech (Mountain View, CA). Phorbol-12-myristate-13-acetate (TPA) and phosphospecific ERK1/2 (Thr202/Tyr204) or total ERK1/2 antibody were from Cell Signaling (Danvers, MA).

CELL CULTURE

The normal mouse prostate epithelial progenitor cells (MPEC)-3 was a gift from Dr. Scott Cramer (Wake Forest University, Winston-Salem). The isolation, culture condition, and properties of MPEC3 were described previously [Barclay and Cramer, 2005; Barclay et al., 2008]. Medium was changed every 48 h. Medium in experiments which use serum-free medium contains BSA, gentamycin, and trace elements in DMEM/F12 (50:50). The progenitor origin of MPEC3 cells were verified by their expression of prostate stem cell markers, Sca 1 and CD49f, as well as their ability of multilineage differentiation into organized prostatic ductal structures [Barclay et al., 2008]. Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

CELL PROLIFERATION ASSAY

Relative cell number was assessed using the MTT assay according to the manufacturers instructions. Results were quantified spectro-photometrically using PowerWaveX Microplate Spectrophotometer (BioTek Instruments, Winooski, VT). Results were from three separate experiments with at least 6 wells per experiment for each treatment condition.

CELL TREATMENT FOR ERK1/2 ACTIVITY

Two conditions were used to determine whether 1,25(OH)₂D modifies ERK1/2 activity: (1) serum-containing medium and (2) serum-free medium. Cells were treated by adding 1,25(OH)₂D (final concentration 100 nM) or vehicle directly to the medium to avoid activation of the pathways due to addition of medium. An untreated control which was not manipulated until harvest was included in all experiments. At the end of the treatment period, cells were rinsed twice with CMF-PBS buffer (136.7 mM NaCl, 2.7 mM KCl, 8.0mM Na₂HPO₄, and 1.5 mM KH₂PO₄ pH 7.4) and collected into lysis buffer containing 25 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1% Triton, protease inhibitor cocktail, and phosphatase inhibitor cocktail. The lysate was centrifuged at 12,000g for 10 min and the supernatant used for immunoblot for phosphorylated and total ERK1/2.

CELL TREATMENT FOR PKC α ACTIVATION

PKC α activation was determined by its association with plasma membrane. MPEC3 were transfected with the PKC α -EGFP construct using Lipofectamine 2000 according to the manufacturer's instructions. Twenty-four hours after transfection, cells were rinsed and given serum-free medium (described above) and treated with 100 nM 1,25(OH)₂D or 1 μ M PKC α agonist, TPA, for the indicated times. Following the incubation, cells were rinsed and harvested into hypotonic HES buffer (20 mM HEPES, 1 mM EDTA, and 250 mM Sucrose, pH 7.4) containing protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and phosphatase inhibitor cocktail (Sigma). Cell lysate was prepared using a glass tissue homogenizer. The cell membrane fraction was separated from cytosolic fraction by ultracentrifugation (Beckman OptimaTM TL Ultracentrifuge) of the homogenate at 100,000g for 1 h at 4 °C. The pellet (membrane fraction) was re-suspended in 20 μ l of HES buffer which included 1% of Triton X-100. Samples were frozen at -20°C for immunoblotting for PKC α -EGFP.

IMMUNOBLOT

Total cellular protein concentration from the collected samples was assessed with the BCA assay. Samples were prepared and separated by SDS-PAGE (10% Tris-HCl). The membranes were incubated with blocking buffer followed by incubation with first antibody: 1:500 GFP monoclonal antibody for PKC α -EGFP, 1:1,500 phosphospecific ERK1/2 (Thr202/Tyr204) or 1:2,000 total ERK1/2 antibody. Immunoreactive bands were visualized with horseradish peroxidase-conjugated anti-rabbit antibody and ECL Advance visualization solution (Amersham Biosciences, Piscataway, NJ). Immunoreacting bands were quantified with Fluor-STM Multi-Imager (Bio-Rad, Hercules, CA). ERK1/2 activity was calculated by first correcting the density of immunoreacting bands with the mean of no treatment control in the same blot, and results expressed as the ratio of phosphorylated ERK1/2 over total ERK1/2.

STATISTICAL ANALYSIS

All experiments were completed at least twice with a final *n* of at least four for each treatment. Student's *t*-tests were used to test difference between two groups. Differences were considered significant when $P \leq 0.05$.

RESULTS

The impact of 1,25(OH) $_2$ D on the growth of MPEC3 was examined. 1,25(OH) $_2$ D suppressed MPEC3 cell growth in a time- and dose-dependent manner. A significant inhibitory effect was observed as early as 24 h after 1 nM 1,25(OH) $_2$ D treatment (4% reduction) and a maximum 21% reduction in cell number was observed at 72 h after 100 nM 1,25(OH) $_2$ D treatment (Fig. 1).

The effect of 1,25(OH) $_2$ D on the rapid activation of ERK1/2 was assessed in MPEC3. A non-significant 50% increase in pERK1/2 level was seen after 15 min of 1,25(OH) $_2$ D treatment when cells were cultured in serum-containing medium (Fig. 2A) but no trend towards activation in cells cultured in serum-free medium (Fig. 2B).

The influence of 1,25(OH) $_2$ D on PKC α activity was assessed in MPEC3. Although the PKC α agonist, TPA, stimulated a sixfold increase in membrane associated PKC α in MPEC-3 cells after 15 min. 1,25(OH) $_2$ D treatment had not impact on PKC α activation at any time point examined (Fig. 3).

DISCUSSION

Our data show that 1,25(OH) $_2$ D causes significant growth arrest in a proliferating prostate progenitor cell line but it does so without the need to activate either ERK1/2 or PKC α signaling. Therefore, 1,25(OH) $_2$ D-induced growth arrest does not depend on rapid activation of these signaling pathways in these cells.

ERK1/2 has been shown to be rapidly activated by 1,25(OH) $_2$ D in a variety of cell types, for example, primary cultures of myocytes [Beno et al., 1995; Buitrago et al., 2001a,b], chondrocytes [Beno et al., 1995; Schwartz et al., 2002], osteosarcoma cells [Wu et al., 2007], hepatic ito cells [Beno et al., 1995], VDR+ and VDR- breast cancer cells [Cordes et al., 2006], and promyelocytic NB4 leukemia cell line [Song et al., 1998] in either serum containing or serum free medium. With only one exception, where ERK1/2 activity was rapidly suppressed by 1,25(OH) $_2$ D treatment in the MCF7 breast cancer cell line [Capiati et al., 2004], the majority of published research has shown 1,25(OH) $_2$ D treatment causes a rapid increase in either or both of ERK isoforms. ERK1/2 activation by has been linked with 1,25(OH) $_2$ D-mediated alteration of cell behavior, such as cell growth and differentiation [Marcinkowska et al., 1997; Chen et al., 1999; Galbiati et al., 2002; Capiati et al., 2004]. For example, rapid (3 min)

and transient activation of ERK1/2 is evident in human colonic carcinoma cell line, CaCo-2 cells, treated with 1,25(OH)₂D (100 nM) in serum-containing medium [Chen et al., 1999]. Consistent with a role for ERK1/2 in 1,25(OH)₂D-mediated alterations in cell behavior, a specific inhibitor of the upstream kinase of ERK1/2 (PD 098059) completely blocked 1,25(OH)₂D induced activation of activator protein-1 (AP-1), which induces the differentiation of CaCo-2 [Chen et al., 1999]. Contrary to previous reports our data do not support a link between ERK1/2 activation and 1,25(OH)₂D mediated growth arrest in the prostate epithelial progenitor cell.

PKC α is another kinase that has been shown to be activated rapidly by 1,25(OH)₂D treatment in a variety of cell types [Balogh et al., 2000; Capiati et al., 2000; Rivera-Bermudez et al., 2002; Boyan et al., 2003; Schwartz et al., 2003; Wali et al., 2003]. In contrast to these reports showing rapid activation of PKC α by 1,25(OH)₂D, our study did not show this effect in prostate epithelial progenitor cells. These indicates that 1,25(OH)₂D-mediated activation of PKC α is not required for growth suppression in normal prostate epithelial progenitor cells.

It is not clear why 1,25(OH)₂D treatment does not stimulate ERK or PKC signaling in the prostate epithelial cell progenitor. Several possible explanations exist. First, there may be cell type specific differences in the expression of MARRS, an alternate membrane associated 1,25(OH)₂D receptor that has been shown to mediate rapid responses of 1,25(OH)₂D [Khanal and Nemere, 2007]. In addition, while Huhtakangas et al. (2004) found that the traditional VDR may mediate membrane signaling pathways by associating with caveolae [Huhtakangas et al., 2004], not all tissues have high caveolae-associated VDR levels (e.g., kidney = liver > heart » lung = duodenum). It is not clear whether VDR exists in the caveolae of prostate epithelial progenitor cells. Future studies will need to be conducted to determine whether alterations in the cellular status or location of the traditional VDR or the MARRS protein exist in prostate epithelial progenitor cells.

Lack of rapid activation of ERK1/2 and PKC α indirectly strengthens the importance of the traditional, genomic signaling pathway of 1,25(OH)₂D as a mediator of growth arrest. Activation of VDR by 1,25(OH)₂D leads to the induction of transcription. A number of genes which may mediate the anti-proliferative activity of 1,25(OH)₂D have been shown, including insulin-like growth factor binding protein (IGFBP)-3 [Stewart and Weigel, 2005], cell cycle cyclin-dependent kinase inhibitory protein (CDKIs), p21 [Liu et al., 1996; Zhuang and Burnstein, 1998; Rao et al., 2004; Saramaki et al., 2006]. Based on our results, we believe that future studies should focus on identifying direct VDR target genes that are crucial for vitamin D-mediated growth arrest.

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REFERENCES

- Balogh G, Boland R, de Boland AR. 1,25(OH)₂-vitamin D₃ affects the subcellular distribution of protein kinase C isoenzymes in rat duodenum: Influence of aging. *J Cell Biochem* 2000;79:686–694. [PubMed: 10996858]
- Barclay WW, Cramer SD. Culture of mouse prostatic epithelial cells from genetically engineered mice. *Prostate* 2005;63:291–298. [PubMed: 1559944]
- Barclay WW, Axanova LS, Chen W, Romero L, Maund SL, Soker S, Lees CJ, Cramer SD. Characterization of adult prostatic progenitor/stem cells exhibiting self-renewal and multilineage differentiation. *Stem Cells* 2008;26(3):600–610. [PubMed: 18055450]

- Beno DW, Brady LM, Bissonnette M, Davis BH. Protein kinase C and mitogen-activated protein kinase are required for 1,25-dihydroxyvitamin D₃-stimulated Egr induction. *J Biol Chem* 1995;270:3642–3647. [PubMed: 7876102]
- Blutt SE, Allegretto EA, Pike JW, Weigel NL. 1,25-Dihydroxyvitamin D₃ and 9-cis-retinoic acid act synergistically to inhibit the growth of LNCaP prostate cells and cause accumulation of cells in G₁. *Endocrinology* 1997;138:1491–1497. [PubMed: 9075707]
- Bonkhoff H. Role of the basal cells in premalignant changes of the human prostate is from a small prostate stem cell population. *Eur Urol* 1996;30(2):201–205. [PubMed: 8875201]
- Boyan SD, Sylvia VL, McKinney N, Schwartz Z. Membrane actions of vitamin D metabolites 1 α , 25(OH)2D₃ and 24R,25(OH)2D₃ are retained in growth plate cartilage cells from vitamin D receptor knockout mice. *J Cell Biochem* 2003;90:1207–1223. [PubMed: 14635194]
- Breitkreutz D, Braiman-Wiksmann L, Daum N, Denning MF, Tennenbaum T. Protein kinase C family: On the crossroads of cell signaling in skin and tumor epithelium. *J Cancer Res Clin Oncol* 2007;133:793–808. [PubMed: 17661083]
- Buitrago C, Boland R, de Boland AR. The tyrosine kinase c-Src is required for 1,25(OH)₂-vitamin D₃ signalling to the nucleus in muscle cells. *Biochim Biophys Acta* 2001a;1541:179–187. [PubMed: 11755212]
- Buitrago C, Vazquez G, De Boland AR, Boland R. The vitamin D receptor mediates rapid changes in muscle protein tyrosine phosphorylation induced by 1,25(OH)₂D₃. *Biochem Biophys Res Commun* 2001b;289:1150–1156. [PubMed: 11741312]
- Capiati DA, Vazquez G, Tellez Inon MT, Boland RL. Role of protein kinase C in 1,25(OH)₂-vitamin D₃ modulation of intracellular calcium during development of skeletal muscle cells in culture. *J Cell Biochem* 2000;77:200–212. [PubMed: 10723087]
- Capiati DA, Rossi AM, Picotto G, Benassati S, Boland RL. Inhibition of serum-stimulated mitogen activated protein kinase by 1 α ,25(OH)₂-vitamin D₃ in MCF-7 breast cancer cells. *J Cell Biochem* 2004;93:384–397. [PubMed: 15368364]
- Chen A, Davis BH, Bissonnette M, Scaglione-Sewell B, Brasitus TA. 1,25-Dihydroxyvitamin D₃ stimulates activator protein-1-dependent Caco-2 cell differentiation. *J Biol Chem* 1999;274:35505–35513. [PubMed: 10585423]
- Cordes T, Diesing D, Becker S, Diedrich K, Reichrath J, Friedrich M. Modulation of MAPK ERK1 and ERK2 in VDR-positive and -negative breast cancer cell lines. *Anticancer Res* 2006;26(4A):2749–2753. [PubMed: 16886687]
- Farach-Carson MC, Davis PJ. Steroid hormone interactions with target cells: Cross talk between membrane and nuclear pathways. *J Pharmacol Exp Ther* 2003;307:839–845. [PubMed: 14534365]
- Galbiati F, Polastri L, Gregori S, Freschi M, Casorati M, Cavallaro U, Fiorina P, Bertuzzi F, Zerbi A, Pozza G, Adorini L, Folli F, Christofori G, Davalli AM. Antitumorigenic and antiinsulinogenic effects of calcitriol on insulinoma cells and solid beta-cell tumors. *Endocrinology* 2002;143:4018–4030. [PubMed: 12239113]
- Huhtakangas JA, Olivera CJ, Bishop JE, Zanello LP, Norman AW. The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1 α ,25(OH)₂-vitamin D₃ in vivo and in vitro. *Mol Endocrinol* 2004;18:2660–2671. [PubMed: 15272054]
- Johansen C, Kragballe K, Henningsen J, Westergaard M, Kristiansen K, Iversen L. 1 α ,25-dihydroxyvitamin D₃ stimulates activator protein 1 DNA-binding activity by a phosphatidylinositol 3-kinase/Ras/MEK/extracellular signal regulated kinase 1/2 and c-Jun N-terminal kinase 1-dependent increase in c-Fos, Fra1, and c-Jun expression in human keratinocytes. *J Invest Dermatol* 2003;120:561–570. [PubMed: 12648218]
- Khanal RC, Nemere I. The ERp57/GRP58/1,25D₃-MARRS receptor: Multiple functional roles in diverse cell systems. *Curr Med Chem* 2007;14(10):1087–1093. [PubMed: 17456022]
- Liu M, Lee MH, Cohen M, Bommakanti M, Freedman LP. Transcriptional activation of the Cdk inhibitor p21 by vitamin D₃ leads to the induced differentiation of the myelomonocytic cell line U937. *Genes Dev* 1996;10:142–153. [PubMed: 8566748]
- Maitland NJ, Bryce SD, Stower MJ, Collins AT. Prostate cancer stem cells: A target for new therapies. *Ernst Schering Found Symp Proc* 2006;5:155–179. [PubMed: 17939301]

- Marcinkowska E, Wiedlocha A, Radzikowski C. 1,25-Dihydroxyvitamin D3 induced activation and subsequent nuclear translocation of MAPK is upstream regulated by PKC in HL-60 cells. *Biochem Biophys Res Commun* 1997;241:419–426. [PubMed: 9425286]
- Miller GJ, Stapleton GE, Ferrara JA, Lucia MS, Pfister S, Hedlund TE, Upadhy P. The human prostatic carcinoma cell line LNCaP expresses biologically active, specific receptors for 1 α ,25-dihydroxyvitamin D3. *Cancer Res* 1992;52:515–520. [PubMed: 1370648]
- Morelli S, Buitrago C, Boland R, de Boland AR. The stimulation of MAP kinase by 1,25(OH)(2)-vitamin D(3) in skeletal muscle cells is mediated by protein kinase C and calcium. *Mol Cell Endocrinol* 2001;173:41–52. [PubMed: 11223176]
- Pardo VG, de Boland AR. Tyrosine phosphorylation signalling dependent on 1 α ,25(OH)2-vitamin D3 in rat intestinal cells: Effect of ageing. *Int J Biochem Cell Biol* 2004;36:489–504. [PubMed: 14687927]
- Peehl DM, Skowronski RJ, Leung GK, Wong ST, Stamey TA, Feldman D. Antiproliferative effects of 1,25-dihydroxyvitamin D3 on primary cultures of human prostatic cells. *Cancer Res* 1994;54:805–810. [PubMed: 7508338]
- Rao A, Woodruff RD, Wade WN, Kute TE, Cramer SD. Genistein and vitamin D synergistically inhibit human prostatic epithelial cell growth. *J Nutr* 2002;132:3191–3194. [PubMed: 12368417]
- Rao A, Coan A, Welsh JE, Barclay WW, Koumenis C, Cramer SD. Vitamin D receptor and p21/WAF1 are targets of genistein and 1,25-dihydroxyvitamin D3 in human prostate cancer cells. *Cancer Res* 2004;64:2143–2147. [PubMed: 15026355]
- Rivera-Bermudez MA, Bertics PJ, Albrecht RM, Mosavin R, Mellon WS. 1,25-Dihydroxyvitamin D3 selectively translocates PKC α to nuclei in ROS 17/2.8 cells. *Mol Cell Endocrinol* 2002;188:227–239. [PubMed: 11911960]
- Saramaki A, Banwell CM, Campbell MJ, Carlberg C. Regulation of the human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the vitamin D3 receptor. *Nucleic Acids Res* 2006;34:543–554. [PubMed: 16434701]
- Schwartz GG, Hulka BS. Is vitamin D deficiency a risk factor for prostate cancer? (Hypothesis). *Anticancer Res* 1990;10:1307–1311. [PubMed: 2241107]
- Schwartz Z, Ehland H, Sylvia VL, Larsson D, Hardin RR, Bingham V, Lopez D, Dean DD, Boyan BD. 1 α ,25-dihydroxyvitamin D(3) and 24R,25-dihydroxyvitamin D(3) modulate growth plate chondrocyte physiology via protein kinase C-dependent phosphorylation of extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase. *Endocrinology* 2002;143:2775–2786. [PubMed: 12072413]
- Schwartz Z, Shaked D, Hardin RR, Gruwell S, Dean DD, Sylvia VL, Boyan BD. 1 α ,25(OH)2D3 causes a rapid increase in phosphatidylinositol-specific PLC-beta activity via phospholipase A2-dependent production of lysophospholipid. *Steroids* 2003;68:423–437. [PubMed: 12798493]
- Skowronski RJ, Peehl DM, Feldman D. Vitamin D and prostate cancer: 1,25 dihydroxyvitamin D3 receptors and actions in human prostate cancer cell lines. *Endocrinology* 1993;132:1952–1960. [PubMed: 7682937]
- Skowronski RJ, Peehl DM, Feldman D. Actions of vitamin D3, analogs on human prostate cancer cell lines: Comparison with 1,25-dihydroxyvitamin D3. *Endocrinology* 1995;136:20–26. [PubMed: 7530193]
- Song X, Bishop JE, Okamura WH, Norman AW. Stimulation of phosphorylation of mitogen-activated protein kinase by 1 α ,25-dihydroxyvitamin D3 in promyelocytic NB4 leukemia cells: A structure-function study. *Endocrinology* 1998;139:457–465. [PubMed: 9449611]
- Sprenger CC, Peterson A, Lance R, Ware JL, Drivdahl RH, Plymate SR. Regulation of proliferation of prostate epithelial cells by 1,25-dihydroxyvitamin D3 is accompanied by an increase in insulin-like growth factor binding protein-3. *J Endocrinol* 2001;170:609–618. [PubMed: 11524241]
- Stewart LV, Weigel NL. Role of insulin-like growth factor binding proteins in 1 α ,25-dihydroxyvitamin D(3)-induced growth inhibition of human prostate cancer cells. *Prostate* 2005;64(1):9–19. [PubMed: 15651061]
- Wali RK, Kong J, Sitrin MD, Bissonnette M, Li YC. Vitamin D receptor is not required for the rapid actions of 1,25-dihydroxyvitamin D3 to increase intracellular calcium and activate protein kinase C in mouse osteoblasts. *J Cell Biochem* 2003;88:794–801. [PubMed: 12577313]

- Wu W, Zhang X, Zanello LP. 1alpha,25-Dihydroxyvitamin D(3) anti-proliferative actions involve vitamin D receptor-mediated activation of MAPK pathways and AP-1/p21(waf1) upregulation in human osteosarcoma. *Cancer Lett* 2007;254(1):75–86. [PubMed: 17412493]
- Zhuang SH, Burnstein KL. Antiproliferative effect of 1alpha,25-dihydroxyvitamin D3 in human prostate cancer cell line LNCaP involves reduction of cyclin-dependent kinase 2 activity and persistent G1 accumulation. *Endocrinology* 1998;139:1197–1207. [PubMed: 9492054]

Fig. 1.

1,25(OH)₂D regulation of MPEC3 growth. MPEC3 were treated with 1,25(OH)₂D (D) or vehicle (V) with indicated doses and times. MTT cell growth assay was performed to assess cell viability. Values represent the means of $n = \text{six samples} \pm \text{SE}$. * Indicates statistically significant difference compared to vehicle control at $P < 0.05$.

Fig. 2.

1,25(OH)₂D regulation of ERK1/2 activity in MPEC3. ERK1/2 activity was assessed with phosphospecific and total antibodies. Cells were treated with 100 nM 1,25(OH)₂D (D) or vehicle (V) for indicated time points. 1,25(OH)₂D was added in serum-containing medium (A,B) or serum-free medium (C,D). An untreated control (O), which was not manipulated until harvest, was loaded in duplicate on each gel. A representative immunoblot of ERK1/2 activity in serum-containing medium (A) and serum-free medium (C) are shown. The quantification of the blots in serum-containing are shown in B and serum-free medium in D. ERK1/2 activity was calculated from the density of bands of phospho-ERK1/2 divided by total-ERK1/2, both of which were corrected against no treatment control (O). Data are expressed as a ratio of

ERK1/2 activity of treated samples to vehicle controls. Values represent mean $\pm$ SE, and n = four. There were no significant differences between 1,25(OH)₂D and vehicle treated ERK1/2 activity.

Fig. 3. 1,25(OH)₂D regulation of PKC α activity in MPEC3. MPEC3 were transiently transfected with EGFP-PKC α vector, followed by treatment with vehicle (V), 1,25(OH)₂D (D) (100 nM), TPA (T) (1 μ M, positive control), or DMSO (M) (vehicle for TPA) for time points indicated. Cell membranes were isolated immediately after harvest and used for immunoblot with anti-GFP antibody. A: A representative immunoblot showing membrane associated PKC α at indicated time points after 1,25(OH)₂D or TPA treatment. The quantification of the blots is shown in B. Data are expressed as ratio of treated samples to vehicle controls. Values represent the means of $n = 4$ for each treatment group $\pm$ SE. * Indicates significant ($P \leq 0.05$) difference between

TPA and vehicle (M) treatment. There were no significant differences in PKC α activity between 1,25(OH) $_2$ D and vehicle treated cells.