The Functional Consequences of Cross Talk between the Vitamin D Receptor and ERK Signaling Pathways are Cell Specific

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Footnote

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Abbreviations

1,25-D, 1,25(OH)$_2$D$_3$; VDR, Vitamin D Receptor; RXR, Retinoid X Receptor; ERK, Extracellular Signal-Regulated Kinase; ALP, Alkaline Phosphatase; MEK, Mitogen Activated Protein Kinase Kinase; SRC, Steroid Receptor Coactivator; DRIP, Vitamin D Receptor Interacting Protein; TR, Thyroid Receptor; PR, Progesterone Receptor.
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Summary

The actions of the active metabolite of 1,25(OH)\textsubscript{2}D\textsubscript{3} (1,25-D) are mediated primarily by the vitamin D receptor (VDR), a member of the nuclear receptor family of ligand activated transcription factors. Although their ligands cause transcriptional activation, many of the ligands also rapidly activate cellular signaling pathways through mechanisms that have not been fully elucidated. We find that 1,25-D causes a rapid, but sustained activation of ERK (extracellular signal-regulated kinase) in bone cell lines. However, the effect of ERK activation on VDR transcriptional activity was cell line specific. Inhibition of ERK activation by the MEK inhibitor, U0126, stimulated VDR activity in MC3T3-E1 cells, but inhibited the activity in MG-63 cells as well as in HeLa cells. VDR is not a known target of ERK. We found that the ERK target responsible for reduced VDR activity in MC3T3-E1 cells is RXR\textalpha. MC3T3-E1 cells express lower levels of RXR\textbeta and RXR\textgamma than either HeLa or MG-63 cells. Although overexpression of RXR\textalpha in MC3T3-E1 cells increased VDR activity, U0126 further enhanced the activity. In contrast, overexpression of RXR\textgamma stimulated VDR activity but abrogated the stimulation by U0126. Thus, although 1,25-D treatment activates ERK in many cell types, subsequently inducing changes independent of VDR, the effects of treatment with 1,25-D on the transcriptional activity of VDR are RXR isoform specific. In cells in which RXR\textalpha is the VDR partner, the transcriptional activation of VDR by 1,25-D is attenuated by the concomitant activation of ERK. In cells utilizing RXR\textgamma, ERK activation enhances VDR transcriptional activity.
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Introduction

The active form of vitamin D, 1,25 (OH)\textsubscript{2}D\textsubscript{3} (1,25-D), regulates calcium homeostasis (1-3), bone remodeling (4;5), proliferation (6;7), differentiation (8) and other cellular and physiological processes. Many of the actions of 1,25-D are mediated by an intracellular receptor, the vitamin D receptor (VDR), a member of the steroid/thyroid receptor superfamily of ligand regulated transcription factors (9;10). The activity of VDR is dependent not only on the concentration of receptor and hormone but also on its heterodimer partner, retinoid X receptor (RXR), and the coactivator proteins that bind to the VDR and facilitate transcription of target genes (11).

In addition to its actions as a modulator of transcription through activation of the VDR, 1,25-D can rapidly activate cell signaling cascades independent of a requirement for transcription (12-14). The means by which 1,25-D induces these changes has not been fully elucidated. Rapid activation of extracellular signal-regulated kinases, ERK 1/ERK 2 in NB4 promyelocytic leukemia cells can be induced not only by 1,25-D, but also by analogs that are unable to activate VDR suggesting the possibility of a separate receptor (15). Antibodies to a membrane protein identified by Nemere et al block the ability of 1,25-D to induce rapid calcium uptake and activation of PKC in cartilage cells (16). VDR-/- osteoblasts take up calcium and activate PKC similar to the wild type osteoblasts, implicating proteins other than VDR in these actions (17). In contrast, Gniadecki has described activation of ERK through 1,25-D induced activation of Raf as a result of interactions between VDR and the adaptor protein Shc (18). VDR null osteoblasts do not exhibit ion channel responses in response to 1,25-D (13) and Erben et al have reported
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that deletion of the VDR DNA binding domain also eliminates non-genomic responses (14). Thus some of the rapid actions of 1,25-D may be dependent upon VDR, while others are not.

Nuclear receptor family members including VDR and RXR as well as many of their coactivators, are phosphoproteins whose activities are also regulated by cell signaling pathways (19-27). Thus 1,25-D can modulate VDR activity both through direct binding to VDR as well as by altering the kinase activities within the cell (9;11;12;28). Although VDR has not been reported to be a substrate for ERK, RXRα (one of the three RXR isoforms) (29) is phosphorylated by ERK as are some of the VDR coactivators including SRC-1 (30).

To better understand the functional interactions between VDR and the ERK signaling pathway, we sought to determine whether 1,25-D activates ERK in the osteoblastic cell lines, MG-63 and MC3T3-E1, and to evaluate the effects of ERK on VDR activity. We found that 1,25-D rapidly induced ERK activity and that this activation persisted at 24 hours in both cell lines. Surprisingly, the effects of ERK activation on VDR activity in the two cell lines were very different. Over-expression of Raf-1 (an upstream activator of ERK) reduced VDR activity in MC3T3-E1 cells, but stimulated activity in MG-63 cells. Similarly, inhibition of ERK by the MEK inhibitor, U0126, stimulated VDR activity in MC3T3-E1 cells, but inhibited VDR activity in MG-63 cells as well as in HeLa cells, a cervical carcinoma cell line commonly utilized to study the functions of nuclear receptors. Although coactivators are targets of ERK signaling (30;31), the primary effect
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of U0126 in MC3T3-E1 cells appears to be enhancement of nuclear localization and DNA binding. An examination of the expression of the RXR isoforms revealed that MC3T3-E1 cells expressed lower levels of RXRβ and RXRγ than did either HeLa or MG-63 cells suggesting that, in MC3T3-E1 cells, the VDR may be more dependent upon RXRα, an isoform whose activity is regulated by ERK (29). While U0126 somewhat increased total VDR expression in both MC3T3-E1 and HeLa cells, nuclear localization and DNA binding of VDR were substantially increased in MC3T3-E1 cells but were minimally affected in HeLa cells. Although over-expression of RXRα in MC3T3-E1 cells increased VDR activity, U0126 further enhanced the activity. In contrast, over-expression of RXRγ stimulated VDR activity but abrogated the stimulation of activity by U0126. Thus, in cells in which RXRα is the dominant VDR partner, activation of ERK by 1,25-D reduces the activity of VDR whereas in cells utilizing RXRβ or RXRγ, the activation of ERK enhances the activity of VDR.

**Materials and Methods**

**Materials.** All cell culture reagents were obtained from Invitrogen (Carlsbad, CA). The phospho-ERK (p42/44) antibody was obtained from New England Biolabs (Beverly, MA), the actin antibody from Chemicon International (Temecula, CA), RXRα, RXRβ, RXRγ, SRC-1 and DRIP205/TRAP-220 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and the VDR antibody was obtained from Affinity Bioreagents (Golden, CO). 1, 25 (OH)2D3 (1,25-D) was obtained from Solvay DuPhar (Weesp, The Netherlands). R5020 (promegestone) was obtained from NEN Life Science
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Products (Boston, MA). Triiodothyronine (T₃) and the alkaline phosphatase assay kits were obtained from Sigma Chemical Company (St. Louis, MO). U0126, the MEK-1 and MEK-2 inhibitor, was obtained from Promega Corporation (Madison, WI). All other reagents used were analytical grade.

**Plasmids.** pCR3.1 SRC-1a (26), pLEN PRB (32), VDRE-tk LUC (33), GRE₂-E₁b-LUC (34), IR0 TRE-tk LUC (35) and VDRE-tk CAT (33) were described earlier. The thyroid receptor β (TRβ) expression vector (a gift from Dr. David Lonard, Baylor College of Medicine, Houston, TX) was made by inserting the TRβ cDNA into the EcoRI site in the pCR3.1 vector. The VDR expression plasmid was a gift from Dr. Wesley Pike, University of Wisconsin, Madison, WI (36). The Raf-1 and empty vector SRα3 plasmids were kind gifts from Dr. Bing Su, University of Texas, M.D. Anderson Cancer Center, Houston, TX. The vitamin D receptor interacting protein (DRIP205) expression plasmid was a kind gift from Dr. Leonard P. Freedman, Memorial Sloan Kettering Research Center, New York, NY (37). RXRα and RXRγ expression plasmids were kind gifts from Dr. David J. Mangelsdorf, University of Texas Southwestern Medical Center, Dallas, TX and Dr. Ronald Evans, Salk institute, La Jolla, CA (38).

**Cell Culture.** MG-63 (human osteoblastic osteosarcoma cell line), HeLa (human cervical carcinoma cell line), MC3T3-E1 (clonal osteoblastic cell line) and CV-1 (green monkey kidney cell line) cells from ATCC were plated in DMEM + 10% charcoal stripped serum with penicillin/streptomycin (Invitrogen) at 200,000 cells per well in six well plates and at 1 million cells per 10 cm dish. For phospho-ERK westerns, the cells were incubated
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for 72 hours in serum free medium to reduce the basal phosphorylation and then treated with vehicle or the indicated concentrations of 1,25-D. Basal levels of phospho-ERK were measured in cells incubated in DME supplemented with 10% charcoal stripped serum for 2 days to mimic the level at the end of transfection studies. To differentiate MC3T3-E1 cells, the cells were plated at a density of 1.7 million cells per 10 cm dish in MEM + 10% serum with 10 mM β-glycerophosphate and 50 µg/ml ascorbic acid for 28 days. Medium was changed every third day. At the end of 25 days, the cells were plated in MEM + 10% charcoal stripped serum and then treated as described in the figures. An increase in the basal alkaline phosphatase activity was taken as an indicator of differentiation.

**Transient Transfection.** Transient transfection of the cells was carried out as described earlier using lysine coupled inactivated adenovirus as non-covalent carriers of the plasmids (39). For each well of a six-well plate, the indicated amount of plasmid DNA was mixed with HEPES buffered saline (0.15 M NaCl, 0.02 M HEPES pH 7.2), and then incubated with $10^8$ virus particles. Thirty minutes later, additional poly-L-lysine (1.3 µg poly-L-lysine/µg DNA) was added to shrink the DNA on the surface of the virus. The virus-DNA complex mixture was added to cells and allowed to infect the cells for 2 h in medium lacking serum, following which the medium was supplemented with charcoal-stripped serum to a final concentration of 5%, and the infection was allowed to continue for 24 hrs. The cells were treated with hormone for an additional 24 hrs, harvested, and assays performed.
**Reporter Gene Assays.** The cells were harvested by incubating in TEN (0.15 M NaCl, 0.01 M EDTA, 0.04 M Tris pH 8.0) at room temperature for 30 min. The cells were pelleted at 13,000 rpm for 30 sec in an Eppendorf 5415C table top centrifuge. Protein from the pelleted cells was extracted with 1X reporter lysis buffer (Promega) containing 0.4 M NaCl for 30 min at room temperature. Luciferase assays were performed using the luciferase assay reagent from Promega Inc. and a Monolight 2010 Luminometer (Analytical Luminescence Lab, Ann Arbor, MI). The luciferase values were normalized to the total protein levels in the cells as measured by the Bradford assay (Biorad, Hercules, CA). Chloramphenicol acetyl transferase assays were performed as described earlier (40) and normalized to total cellular protein.

**Determination of Alkaline Phosphatase activity.** The cells were rinsed once in 1X phosphate buffered saline (PBS) and then scraped in PBS. The cells were then pelleted for 30 sec at 13,000 rpm at 4°C. The cell pellets were suspended in 250 mM Tris (pH 7.5) containing protease inhibitors (1 µg/ml leupeptin, antipain, aprotinin, benzamidine HCl, chymostatin, and pepstatin) and lysed by three cycles of freeze-thaw. The lysates were centrifuged and the supernatant was assayed for alkaline phosphatase activity (41) using an alkaline phosphatase kit from Sigma.

**Western Analysis.** The cells were rinsed once with cold PBS and then scraped in PBS. The cells were then pelleted and extracted in lysis buffer [homogenization buffer (0.05 M potassium phosphate pH 7.5, 10 mM sodium molybdate, 50 mM sodium fluoride, 2 mM EDTA, 2 mM EGTA and 0.05% monothioglycerol) + protease inhibitors (1 µg/ml...
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aprotinin, leupeptin, antipain, benzamidine HCl, pepstatin), 0.2 mM phenyl methyl sulphonyl fluoride and 1 mM sodium vanadate] by three freeze thaw cycles. If the protein extracts that were used for reporter gene assays were also subjected to western analysis, the proteins were extracted in 1X lysis buffer with 0.4 M NaCl to extract the nuclear proteins. The cell debris was pelleted and protein levels were measured by Bradford. Equal amounts of protein extracts were run on an SDS PAGE gel and the proteins were transferred overnight to nitrocellulose at 150 mAmp. After transfer, the membrane was blocked in 1% milk in TBST [1X TBS (Tris Buffered Saline: 10 mM Tris HCl + 150 mM NaCl pH 7.5) + 0.1% Tween 20] for 1 hr for RXR isoforms and actin westerns or incubated in 4 M urea for 3 hrs at room temperature for VDR or pERK westerns. The blots were washed 3 times in 1X TBST 5 min per wash and then incubated with the primary antibody in 1% milk in 1X TBST over night at 4°C. The blots were washed, then incubated for 2 hrs at room temperature with a rabbit anti-rat antibody (Zymed Inc. San Francisco, CA) in 1% BSA in 1X TBST for VDR westerns and with a rabbit anti-mouse antibody (Zymed Inc.) in 1% BSA in 1X TBST for pERK or actin westerns. The blots were washed as described above and the VDR, actin, pERK and the RXR blots were incubated in an anti-rabbit horseradish peroxidase tagged antibody (Amersham Pharmacia Biotech.) in 1X TBST for 1 hr. The blots were washed as described above and the signals were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

SRC-1 and DRIP205 westerns were performed by incubating the blots in 5% milk in 1X TBST overnight at 4°C. The blots were then incubated with the primary antibody for 2
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hrs at room temperature, washed as indicated above and incubated with the anti-rabbit horseradish peroxidase antibody for 1 hr at room temperature. The blots were washed and the signals detected by enhanced chemiluminescence as described above.

**Nuclear Extract Preparation.** Nuclear extracts were prepared from MC3T3-E1 or HeLa cells treated with 20 µM U0126, 10 nM 1,25-D, a combination of U0126 and 1,25-D, or with vehicle for 24 hrs, as described earlier (42). Briefly, the cells were washed once in the dishes with 1X PBS, scraped into 10 ml of PBS, centrifuged at 2000 rpm for 10 min at 4 °C and resuspended in 1 ml PBS. After centrifuging at 4000 rpm for 35 sec at 4 °C, the cells were resuspended in 300 µl of buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM dithiothreitol). After incubating for 10 min on ice to allow swelling, the cells were homogenized with 6 strokes of a Dounce homogenizer and centrifuged at 4000 rpm for 45 sec at 4 °C. The nuclear pellets were gently resuspended in 50-80 µl of buffer C (20 mM HEPES pH 7.9, 400 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, and 0.5 mM dithiothreitol), and homogenized with 8-10 strokes of the Dounce homogenizer. After 30 min incubation on ice, the homogenates were centrifuged at 4500 rpm for 1 min at 4 °C. The nuclear extracts (supernatants) were aliquoted, frozen immediately in a dry ice/ethanol bath and stored at –80 °C for further analysis.

**Electrophoretic mobility shift assay (EMSA).** EMSAs were performed as previously described (43). Binding reactions contained 20 mM Tris–HCl (pH 7.5), 60 mM KCl, 5 mM MgCl₂, 1 mM DTT, 4% glycerol, 100 µg/ml bovine serum albumin (BSA), 25 ng
poly(dI-dC)-poly(dI-dC) (Amersham Biotech., Inc., Piscataway, NJ) as a non-specific competitor, 10,000 cpm of 5' end-labeled DNA (0.003–0.01 ng) and 3.75 µg of nuclear extract in a final volume of 25 µl. Some binding reactions were carried out in the presence of an anti-VDR antibody (Affinity Bioreagents; concentration 1 µg/µl), which was preincubated with nuclear extract in the presence of binding buffer for 20 min at 25 °C. Non-specific competitor and probe DNA were then added, and incubation was carried out for a further 40 min. The binding reactions were fractionated through a native 5% polyacrylamide gel (29% acrylamide, 1% bis-acrylamide in 0.5 X TBE), which was autoradiographed with an intensifying screen at –70 °C. The vitamin D response element of the mouse osteopontin gene (VDRE\textsubscript{mop}) was used as the end-labeled DNA probe. The sequence of the upper strand is shown; the underlined sequences represent the two-hexanucleotide motifs from the mouse osteopontin gene VDRE.

%  \begin{verbatim}
VDRE\textsubscript{mop}   -760  AGAGCAACAAAGGTTCACGAGGTTCACGTCTC -730
\end{verbatim}

Processing of cells for deconvolution microscopy. All steps were performed at room temperature according to the protocol described earlier (44). The cells were plated on coverslips at 200,000 cells per well in a 6 well dish in DMEM supplemented with 10% stripped serum. After treatment, the cells were fixed in 4% formaldehyde (Polysciences Inc., Warrington, PA) in PEM buffer (80 mM Potassium PIPES pH 7.5, 5 mM EGTA and 2 mM MgCl\textsubscript{2}) for 30 min. The cells were then washed 3 times (5 min per wash) in PEM buffer and incubated for 10 min in 0.1 M ammonium chloride to quench autofluorescence. The cells were then washed 2 times (5 min per wash) in PEM buffer and incubated in PEM + 0.5% triton X-100 for 30 min to permeabilize the cells. After washing
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3 times with PEM buffer, the cells were blocked with 5% milk in TBST for 30 min and incubated with the VDR antibody (0.5 µg/ml) in blocking solution for 1 hr. The cells were then washed 3 times (5 min per wash) in TBST and incubated with goat anti-rat Alexa fluor 498 antibody (Molecular Probes, Eugene, Oregon) for 1 hr at room temperature. The cells were washed and fixed again as described above and were counterstained for 1 min with 4, 6-diamidino-2-phenylindole (DAPI) (1 µg/ml) in TBST and mounted in Slow Fade reagent (Molecular Probes, Inc., Eugene, OR).

**Deconvolution microscopy.** Deconvolution microscopy was performed on the processed and mounted coverslips with a Zeiss AxioVert S100 TV microscope (Carl Zeiss, Thornwood, NY) and a Delta Vision Restoration Microscopy System (Applied Precision, Inc.). A Z-series of focal planes were digitally imaged and deconvolved with the Delta Vision constrained iterative algorithm to generate high-resolution images (44). A total of 200 cells were counted and % cells with VDR distributed between nucleus and cytoplasm was determined and expressed in the form of a table.

**RNA Isolation and Northern Hybridization.** Total RNA was isolated from MG-63 and MC3T3-E1 cells using Trizol (Life Technologies). Twenty micrograms of total RNA was separated by 6.65% formaldehyde-1% agarose gel electrophoresis transferred to a nylon membrane (Hybond N+, Amersham Pharmacia), ultraviolet cross-linked, and then hybridized to labeled DNA probes for collagen I-α1 (from the ATCC). Denatured probes were labeled with [³²P]dCTP (Amersham Pharmacia) at 37°C for 15 min using a Random Prime Labeling Kit (Roche). The membranes were prehybridized for 3 h at 65°C and
hybridized with the prepared probe overnight at 65°C in Church hybridization solution (7% SDS, 1% BSA, 0.001 M EDTA and 0.5 M sodium phosphate pH 7). The blots were washed for 30 min each twice at 37°C, once at 65°C for 30 min with wash buffer (1% SDS, 0.05 M sodium phosphate pH 7, 0.01 M EDTA), and then exposed to film. The message levels were quantified using a Storm 860 PhosphorImager equipped with Imagequant software (Molecular Dynamics, Piscataway, NJ).

**Real Time Reverse Transcriptase PCR Analysis.** The RNA from MG-63 cells was diluted 100 fold for 24-hydroxylase rtPCR. Since 24-hydroxylase expression was very low in MC3T3-E1 cells, the RNA from MC3T3-E1 cells was used directly without dilution. RNA from MG-63 and MC3T3-E1 cells were diluted 1500 fold for 18S ribosomal RNA detection. The message was analyzed using real time PCR (ABI PRISM 7700 sequence detector, Applied Biosystems, Foster City, CA) using one step real time rtPCR mix (Applied Biosystems) with TaqMan primers and probes for 24-hydroxylase (human 24-hydroxylase: forward primer- CCCAGCGGCTGGAGATC, reverse primer- CCGTAGCCTTCTTTCGGG, Probe- AACCGTGGAAGGCCTATCGCGACT, mouse 24-hydroxylase: forward primer- TCATTGCGGCCATCAAAAC, reverse primer- TTGGTGTGGAGGCGGCTTGT, probe- ATGAGCACATTTGGGAAGATGATGGTGA from Biosource International, Denver, CO), and a Taqman primer probe set for 18S rRNA from Applied Biosystems. The rtPCR was performed under the conditions of 48°C for 30 min, 95°C for 10 min and 40 cycles of 60°C for 1 min.
All experiments were performed at least three times. The ALP and transient transfection assays were performed in triplicates each time. The ALP, collagen I-α1 and 24-hydroxylase levels were statistically analyzed by one way ANOVA and when significance was revealed (P<0.05), a Holm Sidak-post hoc test was done to identify differences between the groups using Sigma Stat software. All data are represented as mean ± standard error.

**Results**

**1,25-D rapidly and stably activates ERK (ERK1 and ERK2).** Previous reports have shown that 1,25-D induces ERK activation in NB4 promyelocytic leukemia cells (15). To determine whether 1,25-D has a similar effect in bone cells, MG-63 cells, a differentiated human osteoblastic osteosarcoma cell line and MC3T3-E1 cells, an undifferentiated clonal osteoblastic cell line, were used. In charcoal stripped serum MC3T3-E1 cells have higher basal levels of activated ERK than do MG-63 cells (fig. 1B). To study the effects of 1,25-D, the cells were serum starved for 72 hours to reduce basal levels of activated ERK and then treated with 10 nM 1,25-D for 15 min or 24 hrs and phosphorylation (activation) of ERK measured by western blotting. As shown in the fig. 1A, 1,25-D induces the phosphorylation of ERK rapidly (15 min) and its activity remains elevated at 24 hrs.

**Activation of the ERK pathway by constitutively active Raf-1 inhibits VDR activity in MC3T3-E1 cells but stimulates activity in HeLa or MG-63 cells.** To determine
whether increased ERK activity stimulates VDR transcriptional activity, MG-63, MC3T3-E1 and HeLa cells were cotransfected with luciferase or CAT reporter constructs driven by VDREs upstream of the reporter gene and a constitutively active Raf-1 or its empty vector SRα3. Strikingly, activation of the ERK pathway by Raf-1 inhibited the VDR activity in MC3T3-E1 cells but increased activity in HeLa and MG-63 cells (fig. 2).

**Cell type specific regulation of VDR function by an inhibitor of ERK activation.** To measure the effect of the endogenous ERK activity on VDR function, MG-63, MC3T3-E1, and HeLa cells were transfected with VDR responsive reporters and CV-1 cells, which do not express VDR, were cotransfected with a VDR expression plasmid and the luciferase reporter. The cells were then treated or not with U0126, a highly selective and specific inhibitor of MEK1 and MEK2, the kinases that activate ERK (45), in the presence or absence of 1,25-D, and the reporter gene activity measured. Inhibition of MEK by U0126 reduced the VDR activity in MG-63, CV-1 and HeLa cells (fig. 3A), but consistent with the differential response to Raf-1, increased the VDR activity in MC3T3-E1 cells.

A direct comparison of the basal expression of VDR in the VDR expressing cell lines shows that MG-63 and HeLa cells express higher levels of VDR than do the MC3T3-E1 cells (fig. 3B). The differential response, however, was not due to differential regulation of VDR expression. The VDR levels in all three cell lines were increased by 1,25-D treatment presumably due to receptor stabilization as observed previously (46) (fig. 3C).
Surprisingly, treatment of cells with a combination of U0126 and 1,25-D further increased the VDR levels in all cell lines.

**Inhibition of MEK increases the alkaline phosphatase (ALP) activity in undifferentiated and differentiated MC3T3-E1 cells but inhibits activity in MG-63 cells.** To determine whether the MEK inhibitor would also show cell line specific effects on the induction of endogenous target genes, the induction of alkaline phosphatase (ALP) activity by 1,25-D was measured in the presence or absence of U0126. ALP is a direct target of 1,25-D and treatment of cells with 1,25-D increases the ALP activity and subsequent differentiation of cells towards more mature osteoblasts (47;48). Treatment of MG-63 cells with 1,25-D increased the total cellular ALP activity and this activity was inhibited by U0126 (fig. 4A) reproducing the results seen with a transient target. In contrast, in MC3T3-E1 cells alkaline phosphatase (ALP) activity was marginally increased by 1,25-D treatment, but the combination of U0126 and 1,25-D was very effective in inducing ALP activity (fig. 4A).

Since MC3T3-E1 cells are relatively undifferentiated (49), we considered the possibility that the differentiation status of the cells might be responsible for the difference in the VDR function between the MC3T3-E1 and MG-63 cells. Hence, we differentiated the MC3T3-E1 cells in the presence of β-glycerophosphate and ascorbic acid for 28 days and then treated the cells with 1,25-D with or without U0126. As shown in the right panel of fig. 4A, although basal ALP levels were higher in the differentiated cells, treatment with
either 1,25-D or U0126 alone failed to increase the ALP activity. However, treatment of cells with a combination of 1,25-D and U0126 significantly increased the ALP activity.

**Inhibition of MEK increases collagen I-α1 and 24-hydroxylase mRNA in MC3T3-E1 cells but inhibits induction in MG-63 cells.** Since MC3T3-E1 is an undifferentiated cell line and ALP is a differentiation marker, we measured the mRNA levels of VDR target genes not involved in differentiation, collagen I-α1 and 24-hydroxylase, by Northern hybridization and real time rtPCR as described in the methods. Earlier studies have shown that 1,25-D increases 24-hydroxylase and collagen I-α1 mRNA levels in MG-63 cells (50-52). However, in MC3T3-E1 cells collagen I-α1 transcription is resistant to 1,25-D treatment (53). Consistent with the results seen with ALP and with the transient transfection studies, the 1,25-D dependent induction of collagen I-α1 and 24-hydroxylase mRNAs in MG-63 cells was reduced by the MEK inhibitor, U0126 (fig. 4B and 4C). Similar to the earlier report (53), 1,25-D did not induce collagen I-α1 mRNA in MC3T3-E1 cells (fig. 4B). However, pre-treatment of cells with U0126 caused a significant 1,25-D induction of collagen I-α1 mRNA. The combination of U0126 and 1,25-D also increased the 24-hydroxylase mRNA levels in MC3T3-E1 cells significantly more than the 1,25-D treatment alone (fig. 4C). These results suggest that the ERK pathway cell type specifically modulates transcriptional activity of VDR.

**Differential regulation of VDR activity in MC3T3-E1 cells is unique to VDR.** Nuclear receptors share many common coactivators whose activity can be influenced by cell signaling. To determine whether the differential response to inhibition of ERK
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signaling is common to all nuclear receptors, we transfected both HeLa and MC3T3-E1 cells with expression plasmids for thyroid receptor (TR) or for progesterone receptor (PR) and corresponding reporter plasmids and studied the effect of U0126 on the respective receptor activities. As shown in fig. 5, inhibition of the ERK pathway reduced TR activity in both HeLa (fig 5D) and MC3T3-E1 (fig. 5A) cells and did not significantly alter the activity of PR in either cell line (fig. 5E and 5B). Thus, the differential response is not due to factors common to all nuclear receptor dependent transcription.

Stimulation of VDR activity by DRIP205 and SRC-1. An earlier report indicated that VDR activity is augmented in undifferentiated keratinocytes by the coactivator DRIP205 and in differentiated keratinocytes by the coactivator SRC-1 (54). Since MC3T3-E1 cells are undifferentiated clonal osteoblasts, we speculated that the difference in VDR activity seen between MC3T3-E1 and other cells could be the role played by different coactivators such as DRIP205 and SRC-1, which are the receptor interacting components of distinct coactivator complexes (37). SRC-1 is a target of ERK and its ability to stimulate AR and PR activity depends in part on phosphorylation of sites in SRC-1 (26;30;55). A comparison of the relative expression levels of SRC-1 and DRIP205 in MC3T3-E1 and HeLa cells revealed that HeLa cells express higher levels of DRIP205 while MC3T3-E1 cells express higher levels of SRC-1 (fig. 6A). Consistent with the relatively low levels of DRIP205, transfection of DRIP205 stimulated VDR activity in MC3T3-E1 cells somewhat more than SRC-1 (fig. 6B). In contrast, SRC-1 and DRIP205 were equally able to stimulate VDR activity in HeLa cells. However, the pattern of VDR response to U0126 was unaffected by transfection of either coactivator (fig. 6C).
Differential expression of RXR isoforms. The failure of the coactivators to reverse the response to U0126 suggested that ERK signaling may influence an earlier step in receptor activation. The most likely candidate is the RXR heterodimer partner of VDR (11;56). An analysis of expression levels revealed that the levels of the three isoforms in HeLa and MG-63 cells are similar but the levels of RXRβ and RXRγ are lower in MC3T3-E1 cells (fig. 7A). In contrast, RXRα levels are more equivalent in the three cell lines. Treatment with 1,25-D reduces the levels of RXRα in MC3T3-E1 cells, but not in the other cell lines (fig. 7B). These data imply that VDR action may be strongly dependent on RXRα in MC3T3-E1 cells. ERK dependent phosphorylation of RXRα causes resistance to 1,25-D in ras transformed keratinocytes (29). Thus, if VDR activity is RXRα dependent in MC3T3-E1 cells, the higher basal level of ERK (fig. 1B) in these cells in combination with the additional ERK activity elicited by 1,25-D should reduce functional heterodimer formation.

Effect of U0126 on DNA Binding and Subcellular Localization. If active RXR is the limiting factor in MC3T3-E1 cells, then treatment with U0126 should enhance VDR binding to DNA as the VDR/RXR heterodimer binds more stably to DNA than does a VDR dimer (57). Thus, to determine whether U0126 treatment altered DNA binding, nuclear extracts were prepared from MC3T3-E1 and HeLa cells treated with U0126 or not in the presence or absence of 1,25-D and mobility shift assays (EMSA) were performed. In HeLa cells a prominent band was observed (fig. 8A) whose strength was reduced by an antibody to the DNA binding domain of VDR, which reduces the binding
of VDR complex to DNA (58). U0126 appeared to have no effect on the binding of the unliganded receptor, but, if anything, modestly reduced the signal in the presence of 1,25-D. In contrast, the DNA binding in the nuclear extract from MC3T3-E1 cells was much weaker and the gel had to be exposed to film for a much longer time to detect DNA binding (compare the intensities of the free DNA bands in the HeLa and MC3T3-E1 experiments). U0126 substantially stimulated the DNA binding both in the absence and the presence of 1,25-D; this binding was reduced, as expected, by the VDR antibody (fig. 8B). U0126 and 1,25-D treatments had modest and comparable effects on total VDR and nuclear VDR levels in HeLa cells (fig. 8C). In contrast, 1,25-D either in the absence or presence of U0126 preferentially increased the amount of VDR in the nuclear extract. Although U0126 alone also increased the amount of total and nuclear VDR, it had a greater effect on DNA binding than did 1,25-D alone. Thus, 1,25-D appears to regulate the partitioning of the receptor between the nuclear and cytoplasmic fractions, but inhibition of ERK activity is required for efficient DNA binding.

**VDR is exclusively nuclear in HeLa cells but distributed between the nucleus and cytoplasm in MC3T3-E1 cells.** Although most studies have reported nuclear localization of VDR, the DNA binding studies suggest that VDR is either not exclusively nuclear in MC3T3-E1 cells, or is less tightly bound to the nucleus. To assess this, receptor distribution in HeLa and MC3T3-E1 cells treated with vehicle, 1,25-D, U0126 or a combination of U0126 and 1,25-D was detected by indirect immunofluorescence. As shown in fig. 9, VDR was exclusively nuclear in all HeLa cells and no change in the receptor localization was seen upon treatment. However, in MC3T3-E1 cells, although
the majority of the VDR was in the nucleus some of the receptor was found in the cytoplasm. A count of cells with exclusively nuclear VDR compared to those that displayed some cytoplasmic localization revealed that 47% displayed the partial cytoplasmic localization depicted in the figure (lower table of fig. 9). However, treatment of cells with either U0126, 1,25-D or a combination of 1,25-D and U0126 translocated all of the VDR into the nucleus.

**Overexpression of RXRγ but not RXRα eliminates U0126-dependent increases in VDR activity in MC3T3-E1 cells.** To determine whether expression of RXR isoforms can alter the response to U0126, MC3T3-E1 cells were transfected with plasmids encoding, RXRγ, RXRα or empty vector. The cells were treated with vehicle, 1,25-D, U0126 or a combination of U0126 and 1,25-D. Overexpression of either RXRα or RXRγ in MC3T3-E1 cells increased the VDR activity (fig. 10). In cells transfected with empty vector or RXRα, the MEK inhibitor, U0126, further enhanced the VDR activity. However, in cells transfected with RXRγ, the MEK inhibitor was unable to further stimulate the VDR activity (fig. 10). Thus the heterodimer partner of VDR is the determining factor in the sensitivity of VDR to ERK signaling.

**Discussion**

Recent studies have shown that rapid activation of cell signaling pathways (including pathways leading to p42/p44 ERK activation) is a fairly common response to steroids such as estrogen (59), progesterone (60), and androgen (61). Similarly, 1,25D has rapid
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effects on the activity of cell signaling pathways. The increased activity of the cell signaling pathways can also regulate the nuclear activities of the hormone receptors and their coactivators. We found that 1,25-D rapidly induces activation of p42/p44 ERK in MG-63 and MC3T3-E1 cells and the activation is still evident at 24 hours (fig. 1). However the consequences of the activation on VDR activity in the two cell lines differed markedly. In MC3T3-E1 cells activation of ERK reduces the subsequent genomic activity of VDR whereas the genomic activity of VDR is enhanced by ERK in the other cell lines. Transfection with Raf-1, an upstream activator of p42/p44 ERK inhibited VDR activity in MC3T3-E1 cells, but enhanced activity in MG-63 cells, whereas inhibition of kinase activation by U0126 had the opposite effect. Despite this difference, U0126 treatment enhanced expression of VDR in both cell lines (fig. 3). Although previous studies in keratinocytes suggested a potential for differential coactivator usage as a function of differentiation (54), and coactivators are phosphoproteins, more highly differentiated MC3T3-E1 cells responded similarly to the undifferentiated cells. Supplementation with either SRC-1 or DRIP205, two VDR coactivators, had no effect on the response to the ERK pathway.

Another potential regulatory point is nuclear localization and subsequent DNA binding. Neither U0126 nor 1,25-D had much effect on the ability of HeLa cell VDR to bind to a VDRE in an EMSA assay (fig. 8). In marked contrast, U0126 strongly stimulated DNA binding of VDR from nuclear extracts of MC3T3-E1 cells (fig. 8). In HeLa cells, the levels of VDR in the nuclear extracts reflected the levels in whole cell extracts. In MC3T3-E1 cells, the increase in the nuclear extract (material tightly bound in the
nucleus) was much greater upon 1,25-D treatment than in the total cellular extract. Immunocytochemical studies of VDR localization generally show that VDR is nuclear in the absence of hormone (62). However, recent studies show that VDR-RXR shuttles between the cytoplasm and nucleus (63). To determine whether the relative distribution of VDR differs in MC3T3-E1 cells, we looked at the distribution of VDR in fixed cells and found that, in contrast to HeLa cells, some of the VDR is cytoplasmic in about 40% of the MC3T3-E1 cells examined and that treatment with either 1,25-D or U0126 causes the receptor to be localized to the nucleus (fig. 9). MC3T3-E1 cells express higher basal levels of activated ERK (fig. 1) and this may contribute to the differential distribution of VDR in the two cell lines in the absence of treatment.

The increase in DNA binding in MC3T3-E1 cells may, in part, be due to increased nuclear levels of VDR, but the binding is not strictly proportional to VDR expression as 1,25-D treatment alone was less effective than U0126 in increasing DNA binding despite the higher levels of VDR (fig. 8). The lack of correlation between nuclear VDR levels and DNA binding suggested that the VDR heterodimer partner, RXR, might be limiting. An analysis of RXR isoform expression levels revealed that MC3T3-E1 cells had lower levels of RXRβ and RXRγ than did HeLa or MG-63 cells. Previous studies have shown that RXRα is a substrate for ERK (29) and there is evidence that phosphorylation of this site reduces the effectiveness of RXRα as a VDR partner. Ras transformed keratinocytes are resistant to 1,25-D mediated differentiation (64) and this resistance is due to the phosphorylation of Ser260 of RXR (29). Mutation of this serine to alanine eliminated the resistance. Interestingly, the response of TR, which also forms heterodimers with RXR,
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was not differentially affected by ERK signaling in the two cell lines (fig. 5). Consistent with the correlation with the levels of RXR isoforms, elevated expression of RXRγ eliminated the U0126 stimulation of VDR activity. Thus, although coactivator function may be modified by ERK signaling, the dominant determinant of the effect of ERK signaling on VDR function is its RXR partner. In cells in which RXRα predominates, the elevation of ERK signaling by 1,25-D likely blunts the VDR transcriptional responses whereas in cells containing higher levels of the other isoforms, the enhanced ERK signaling stimulates VDR activity. The RXR isoform dependent response indicates that depending upon the cellular milieu, treatment with 1,25-D will activate ERK with little activation of the transcriptional activity of VDR (RXRα dominant cells) whereas in other cells (RXRβ or RXRγ dominant), 1,25-D potentiates VDR activity both through its action as a ligand as well as through activation of ERK. ERK activity is often associated with cell growth (65-67) whereas 1,25-D acting through VDR often inhibits growth and induces differentiation (6-8) although 1,25-D stimulates proliferation in some cell types. Thus, the relative abundance of RXR may play a role in determining the extent to which 1,25-D alters proliferation.

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Figure Legends

Figure 1: 1,25-D rapidly and stably activates extracellular signal-regulated kinase (ERK) in MG-63 and MC3T3-E1 cells. A. MG-63 or MC3T3-E1 cells were serum starved for 72 hrs to reduce basal levels of p42, 44 ERK and then treated with vehicle or 10 nM 1,25-D for 15 min or 24 hrs. The cell extracts were run on a 12.5% SDS-PAGE gel and western blotted with a phospho specific ERK antibody as indicated in the methods. The blots were stripped and re-probed with an antibody to actin as a loading control. B. Basal levels of p42, 44 ERK were determined by maintaining the cells in medium containing charcoal stripped serum for 48 hrs (to mimic the levels present in transfection studies) and then pERK detected as above. p42, p44, 42 and 44 KDa ERK proteins; 1,25-D, 1,25 (OH)2D3.

Figure 2: Activation of ERK by constitutively active Raf-1 increases VDR activity in MG-63 and HeLa cells but inhibits VDR activity in MC3T3-E1 cells. HeLa and MC3T3-E1 cells were transfected with 0.25 µg VDRE-tk LUC; MG-63 cells were transfected with 0.25 µg VDRE-tk CAT. All cells were also transfected with 0.15 µg of either the empty vector SRα3 or constitutively active Raf-1. One day after transfection cells were treated with vehicle or 10 nM 1,25-D for an additional 24 hrs, harvested, luciferase and CAT activity measured and normalized to total cellular protein. CAT, chloramphenicol acetyl transferase; RLU, relative light units.

Figure 3: Inhibition of MEK increases the VDR activity in MC3T3-E1 but not in HeLa, MG-63 or CV-1 cells. A. MC3T3-E1, CV-1 and HeLa cells were transfected with...
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0.25 µg VDRE-tk LUC as described in the methods. CV-1 cells were transfected with 5 ng vitamin D receptor (VDR) in addition to the VDRE-tk LUC. MG-63 cells were transfected with 0.25 µg of VDRE-tk CAT. Twenty four hours after transfection, the cells were treated with 20 µM U0126 or 10 nM 1,25-D or pre-treated with U0126 and 30 min later treated with 1,25-D. The cells were harvested 24 hrs after treatment and luciferase activity measured and normalized to total cellular protein levels. B. Relative basal levels of VDR in HeLa, MG-63 and MC3T3-E1 cells were determined by analyzing equal amounts of cellular extract for VDR and actin expression by western blotting. C. The lysates from the cells treated as indicated in panel A were fractionated on a 12.5% SDS-PAGE and VDR and actin detected by western blotting. CAT, chloramphenicol acetyl transferase; RLU, relative light units.

Figure 4: Inhibition of MEK enhances the 1,25-D dependent induction of alkaline phosphatase (ALP) activity and collagen I-α1 and 24-hydroxylase mRNA in MC3T3-E1 cells but inhibits in MG-63 cells. A. MG-63 or MC3T3-E1 cells were treated with 20 µM U0126 or 10 nM 1,25-D or pre-treated with U0126 and 30 min later treated with 1,25-D for a total of 72 hrs. The cells were harvested and the ALP activity measured and normalized to the total cellular protein levels. Right panel: MC3T3-E1 cells were differentiated as indicated in the methods for 28 days in the presence of β-glycerophosphate and ascorbic acid and treated as described above. ALP activity was measured and normalized to the total cellular protein. B. MG-63 and MC3T3-E1 cells were treated with 20 µM U0126 or 10 nM 1,25-D or pre-treated with U0126 and 30 min later treated with 1,25-D for a total of 24 hrs. RNA isolated from the cells was run on a
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denaturing agarose gel, transferred to nitrocellulose and probed for collagen I-α1 by northern blotting. The message was normalized to 18S. C. RNA from cells treated as indicated for panel B was reverse transcribed and the 24-hydroxylase message detected by real time quantitative PCR using taqMan primers and probe. The 24-hydroxylase message was normalized to 18S. In panels B and C, the level of mRNA in 1,25-D treated cells is set at one. ALP- Alkaline Phosphatase, * significance at P<0.05 from vehicle treated cells. # in panels B and C indicates significance at P<0.05 from 1,25-D treated cells.

Figure 5: Differential regulation of VDR activity by ERK in MC3T3-E1 cells is unique to VDR. MC3T3-E1 cells were transfected with 0.25 µg TRE-tk LUC and 10 ng TR (panel A), 0.25 µg GRE LUC and 10 ng PR (panel B) or 0.25 µg VDRE-tk LUC (panel C) as indicated in the methods. HeLa cells were transfected with 0.25 µg TRE-tk LUC and 10 ng TR (panel D), 0.25 µg GRE LUC and 10 ng PR (panel E) or 0.25 µg VDRE-tk LUC (panel F). Twenty four hours after transfection, the cells were treated with 20 µM U0126 or 10 nM of the indicated hormone or pre-treated with U0126 and after 30 min treated with the indicated hormone for 24 h. The cells were harvested, luciferase activity measured and normalized to the total cellular protein. TR, Thyroid receptor; PR, progesterone receptor; TRE, thyroid response element; GRE. Glucocorticoids/Progesterone response element.

Figure 6: Coactivation of VDR by SRC-1 and DRIP205. A. Protein extracts from MC3T3-E1 and HeLa cells were fractionated on a 6.5% PAGE, transferred overnight and
western blotted with antibodies against SRC-1 and DRIP205. B. MC3T3-E1 and HeLa cells were transfected with 0.25 µg VDRE-tk LUC and with 0.5 µg empty vector or SRC-1 or DRIP205 as indicated in the figure. Twenty-four hours after transfection, the cells were treated with 10 nM 1,25-D for 24 hrs. The cells were harvested, luciferase activity measured and normalized to total cellular protein levels. The results are represented as fold change from the vector-transfected hormone-treated cells. C. MC3T3-E1 and HeLa cells were transfected with 0.25 µg VDRE-tk LUC and 0.5 µg empty vector, SRC-1 or DRIP205 as indicated in the figure, treated with 20 µM U0126, 10 nM 1,25-D, or a combination of both for 24 hrs. The cells were harvested and the luciferase activity measured and normalized to total cellular protein. The results are represented as fold change from the vector transfected hormone treated samples taken as unity. VDR, vitamin D receptor; SRC-1, steroid receptor coactivator-1; DRIP205, D receptor interacting protein-205; RLU, relative light units.

**Figure 7: Differential expression of RXR isoforms.** A. Total cellular extracts prepared from HeLa, MC3T3-E1 and MG-63 cells were run on a 10% SDS-PAGE and blotted with antibodies against RXR-α, RXR-β or RXR-γ. An actin western was performed as a normalization control. B. HeLa, MC3T3-E1 and MG-63 cells were treated with vehicle or 10 nM 1,25-D for 24 hrs. The cells were harvested and the total cellular extracts run on a 10% SDS PAGE and the levels of RXR-α detected by western. Actin was used as a normalization control. The signals were densitometrically quantified and are shown in the lower panel as bar graphs with vehicle treated control taken as unity. RXR- Retinoid X receptor.
**Figure 8: U0126 treatment increases DNA binding in MC3T3-E1 cells.** HeLa (A) and MC3T3-E1 cells (B) were treated as indicated in the figure for 24 hrs. The cells were harvested, nuclear extract prepared and an electrophoretic mobility shift assay (EMSA) performed as described in the methods. VDR 9A7 antibody was used to compete the binding of VDR to DNA. C. Nuclear extracts and total cellular extracts were prepared from MC3T3-E1 and HeLa cells treated with 20 µM U0126, 10 nM 1,25-D or pre-treated with U0126 for 30 min and treated with 1,25-D for 24 h. The extracts were run on a 12.5% SDS-PAGE and then western blotted with a VDR antibody. C-, VDR-DNA complex; NE-, nuclear extract; VDR-Ab- Vitamin D receptor antibody; F-, free probe.

**Figure 9: Localization of VDR in MC3T3-E1 and HeLa cells.** MC3T3-E1 or HeLa cells were plated on coverslips and then treated as indicated in the figure for 24 hrs. The cells were then fixed. VDR was detected using a VDR antibody (green fluorescence) and cell nuclei were detected using DAPI (blue fluorescence). A total of 200 cells were counted for each treatment and the percent of cells displaying VDR both in the cytoplasm and nucleus is reported in the lower panel as a table.

**Figure 10: Over expression of RXR-γ but not RXR-α reverses the increase in VDR activity due to the inhibition of ERK.** MC3T3-E1 cells were transfected with 0.25 µg VDRE-tk LUC and 25 ng RXR-α or RXR-γ expression plasmid or empty vector. Twenty four hours after transfection, the cells were treated with 20 µM U0126, 10 nM 1,25-D or
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a combination of both. Luciferase activity was then measured and normalized to total cellular protein. RXR, retinoid X receptor.
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Figure 1.

A

| Time    | MG-63  | MC3T3-E1 |
|---------|--------|----------|
| 15 min  | -      | -        |
| 24 hrs  | +      | +        |

10 nM 1,25-D

- -

\[ \text{p44} \]

\[ \text{p42} \]

\[ \text{Actin} \]

B

\[ \text{MG-63} \]

\[ \text{MC3T3} \]

\[ \text{p44} \]

\[ \text{p42} \]

\[ \text{Actin} \]
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**Figure 2.**

![Graph showing the effect of 1,25-D on CAT and RLU for MG-63, HeLa, and MC3T3-E1 cells with and without SRα3 and Raf-1 treatments.](image)

- **MG-63**
  - 1,25-D
    - SRα3: -/+, +/+ RLU (x10^3)
    - Raf-1: -/+, +/+ RLU (x10^3)

- **HeLa**
  - 1,25-D
    - SRα3: -/+, +/+ RLU (x10^3)
    - Raf-1: -/+, +/+ RLU (x10^3)

- **MC3T3-E1**
  - 1,25-D
    - SRα3: -/+, +/+ RLU (x10^3)
    - Raf-1: -/+, +/+ RLU (x10^3)
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Figure 3.

A

|        | MG-63 | HeLa |
|--------|-------|------|
| 1,25-D | -     | -    |
| U0126  | +     | -    |
| CAT pmp (x10^3) | 4000 | 1500 |
| RLU (x10^3) | 0    | 0    |

MG-63

|        | CV-1 | MC3T3-E1 |
|--------|------|----------|
| 1,25-D | -    | -        |
| U0126  | +    | -        |
| RLU (x10^3) | 150  | 1500     |

CV-1

B

MG-63 | MC3T3-E1 | HeLa

VDR

C

|        | MG-63 | MC3T3-E1 | HeLa |
|--------|-------|----------|------|
| 1,25-D | -     | -        | -    |
| U0126  | +     | -        | +    |
| VDR    | -     | -        | -    |
| Actin  | -     | -        | +    |
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Figure 4.

A

|          | MG-63 |          |          | MC3T3-E1       |          | MC3T3-E1-Differentiated |
|----------|-------|----------|----------|----------------|----------|------------------------|
| 1,25-D   | -     | -        | +        | -              | +        | -                      |
| U0126    | -     | +        | -        | -              | +        | +                      |

ALP IU/µg Protein

B

|          | Collagen I-α1/18S |
|----------|------------------|
| 1,25-D   | MG-63            |
| U0126    | MC3T3-E1         |

C

|          | 24(OH)ase/18S   |
|----------|-----------------|
| 1,25-D   | MG-63           |
| U0126    | MC3T3-E1        |
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Figure 5.

A

|          | TR   |
|----------|------|
| U0126    | -    |
| T3       | -    |
| R5020    | -    |
| 1,25-D   | -    |

B

|          | PR   |
|----------|------|
| U0126    | -    |
| T3       | -    |
| R5020    | -    |
| 1,25-D   | -    |

C

|          | VDR  |
|----------|------|
| U0126    | -    |
| T3       | -    |
| R5020    | -    |
| 1,25-D   | -    |

D

|          | TR   |
|----------|------|
| U0126    | -    |
| T3       | -    |
| R5020    | -    |
| 1,25-D   | -    |

E

|          | PR   |
|----------|------|
| U0126    | -    |
| T3       | -    |
| R5020    | -    |
| 1,25-D   | -    |

F

|          | VDR  |
|----------|------|
| U0126    | -    |
| T3       | -    |
| R5020    | -    |
| 1,25-D   | -    |
Cross Talk between the Vitamin D Receptor and ERK Signaling

Figure 6.

A

B

C

MC3T3-E1
HeLa
SRP-1
DRIP205
Actin

RLU (Fold change)

0
5
10
15

SRC-1
DRIP205
1,25-D
MC3T3-E1
HeLa

RLU (Fold change)

0
2
4
6
8
10
12
14
16
18
20

1,25-D
U0126
Backbone
SRC-1
DRIP205
MC3T3-E1
HeLa

RLU (Fold change)

0
0.5
1
1.5
2
2.5
3
3.5
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

U0126
Backbone
SRC-1
DRIP205
MC3T3-E1
HeLa

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Figure 7.

A

|        | HeLa | MC3T3-E1 | MG-63 |
|--------|------|----------|-------|
| RXRα   |      |          |       |
| RXRβ   |      |          |       |
| RXRγ   |      |          |       |
| Actin  |      |          |       |

B

|        | HeLa | MC3T3-E1 | MG-63 |
|--------|------|----------|-------|
| 1,25-D | -    | +        | -     |
| RXRα   |      |          |       |
| Actin  |      |          |       |

Graph showing the effect of 1,25-D on RXRα expression in HeLa and MC3T3-E1 cells.
Cross Talk between the Vitamin D Receptor and ERK Signaling

Figure 8.

|   | NE | VDR-Ab | HeLa |
|---|----|--------|------|
|   | -  | +      | +    |
|   | -  | +      | +    |
|   | -  | +      | +    |
|   | -  | +      | +    |
|   | +  | +      | +    |

|   | UO126 | 1,25-D |
|---|-------|--------|
|   | -     | -      |
|   | +     | +      |
|   | -     | -      |
|   | +     | +      |

|   | MC3T3-E1 |
|---|----------|
|   | NE | VDR-Ab |
|   | -  | +      |
|   | -  | +      |
|   | -  | +      |
|   | -  | +      |
|   | +  | +      |
|   | +  | +      |

|   | Nuclear Extract | Total Cellular Extract |
|---|----------------|------------------------|
| U0126 | - | - | + | + |
| 1,25-D | - | + | - | + |

VDR (MC3T3-E1)  
VDR (HeLa)
Cross Talk between the Vitamin D Receptor and ERK Signaling

Figure 9.

Percent Cells containing both nuclear and cytoplasmic VDR

| Treatment            | MC3T3-E1 | HeLa |
|----------------------|----------|------|
| Control              | 47       | 5    |
| 1,25-D               | 3        | 4    |
| U0126                | 4        | 2    |
| U0126+1,25-D         | 3        | 3    |
Figure 10.

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Figure 10.
The functional consequences of cross talk between the vitamin D receptor and ERK signaling pathways are cell specific
Ramesh Narayanan, Veronica A. Tovar Sepulveda, Miriam Falzon and Nancy L Weigel

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