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Vitamin D Receptor Controls Expression of the Anti-aging Klotho Gene in Mouse and Human Renal Cells

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Abstract

Isoforms of the mammalian klotho protein serve as membrane co-receptors that regulate renal phosphate and calcium reabsorption. Phosphaturic effects of klotho are mediated in cooperation with fibroblast growth factor receptor-1 and its FGF23 ligand. The vitamin D receptor and its 1,25-dihydroxyvitamin D₃ ligand are also crucial for calcium and phosphate regulation at the kidney and participate in a feedback loop with FGF23 signaling. Herein we characterize vitamin D receptor-mediated regulation of klotho mRNA expression, including the identification of vitamin D responsive elements (VDREs) in the vicinity of both the mouse and human klotho genes. In keeping with other recent studies of vitamin D-regulated genes, multiple VDREs control klotho expression, with the most active elements located at some distance (–31 kb to –46 kb) from the klotho transcriptional start site. We therefore postulate that the mammalian klotho gene is up-regulated by liganded VDR via multiple remote VDREs. The phosphatemic actions of 1,25-dihydroxyvitamin D₃ are thus opposed via the combined phosphaturic effects of FGF23 and klotho, both of which are upregulated by the liganded vitamin D receptor.

1. Introduction

Alpha-Klotho, hereafter referred to as klotho, is an anti-aging gene expressed predominately in kidney and brain choroid plexus [1], and at lower levels in several other tissues [2]. The full-length, membrane form of klotho (m-KL) consists of two similar domains (termed KL1 and KL2 [3]) with similarity to glycosyl hydrolases, a transmembrane domain and a short intracellular domain. m-KL acts as a coreceptor with fibroblast growth factor receptor-1 (FGFR1)[2] to bind fibroblast growth factor 23 (FGF23) and mediate phosphaturia to correct the hyperphosphatemia arising from 1,25-dihydroxyvitamin D (calcitriol, abbreviated 1,25D) [4] stimulation of intestinal calcium (and phosphate) absorption.

Proteolyzed klotho (p-KL) is generated by cleavage at the short transmembrane domain [5] and is shed into the circulation. The p-KL form has direct enzymatic effects in the proximal

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tubule to modify membrane proteins by removing sialic acid residues [6], thereby affecting TRPV5 and ROMK1 transporters, as well as insulin/IGFI and Wnt signaling [2]

An alternatively spliced klotho transcript encodes a 549-residue peptide in the human [3] and a 550 residue-protein in mouse [7] (Fig. 1). This truncated klotho, if produced, contains a signal peptide without a transmembrane domain, and is herein designated as secreted klotho (s-KL).

The hormonal form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25D), is also proposed to have anti-aging effects [4]. 1,25D actions are mediated through the nuclear vitamin D receptor (VDR) binding directly to vitamin D responsive elements (VDREs) along with its RXR heterodimeric partner [4]. 1,25D-liganded VDR induces FGF23 in osteocytes to boost circulating FGF23 [8], which promotes phosphaturia to protect against hyperphosphatemia [4]. FGF23 also increases 1,25D degradation via induction of CYP24A1, and repress CYP27B1 to curb 1,25D biosynthesis [9]. The present study pursues the hypothesis that 1,25D regulates the expression of both membrane and soluble klotho forms in multiple kidney cell types to support FGF23 phosphaturic and vitamin D counterregulatory actions at the kidney, possibly exerting anti-aging effects.

2. Materials and Methods

2.1 Cell lines and real time PCR

Murine distal convoluted tubule (mpkDCT) cells were cultured as described [10]. All other cell lines, including human embryonic kidney (HEK) cells, murine inner medullary collecting duct (IMCD-3) cells, human proximal tubule (HK-2) cells, and simian kidney (COS-7) cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured as recommended. Culture media, fetal bovine serum, and penicillin-streptomycin stocks were obtained from Gibco (Invitrogen Corp., Carlsbad CA). Crystalline 1,25D was a kind gift from Milan Uskokovic of Hoffmann-LaRoche.

Total RNA was isolated from <2 million cells using an Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. First strand cDNA was synthesized from 1 µg of RNA using an iScript kit (Bio-Rad Hercules, CA) according to the manufacturer's instructions in 20 µl total volume.

Quantitative real time PCR (qPCR) was performed with Applied Biosystems SYBR Green 2X PCR Master Mix (Life Technologies, Carlsbad CA) in a System 7500 Fast thermal cycler using 2 µl of first strand DNA and 1 µl of 18 µM primer mixture in 20 µl total volume.

For detection of human klotho transcripts, the forward primer was 5'-GATAGAGAAAAATGGCTTCCCTCC-3' and the reverse primer was 5'-GGTCGGTAAACTGAGACAGAGTGG-3', which amplify both mRNA spliceforms, yielding a 131 bp product for m-KL and a 181 bp product for s-KL (Fig. 1A). Human CYP24A1 was detected using forward primer 5'-CAGCGAACTGAACAAATGGTCG-3' and reverse primer 5'-TCTCTTCTCATACAACACGAGGCAG-3', and human GAPDH was amplified using 5'-TGACAACTTTGGTATCGTGGAAGG-3' and 5'-AGGGATGATGTTCTGGAGAGCC-3' primers. In some experiments, HK-2 cells were transfected with 150ng of pSG5-hVDR [11] in order to supply exogenous VDR. Mouse klotho transcripts were detected using primers 5'-TGATGTCGTCCAACACGTAGGCTT-3' and 5'-GCAAAGTGCTCAACTGGCTAAGGT-3', which amplify the m-KL spliceform to produce a 135 bp product (Fig. 1B). A second primer pair (5'-TTGCTGGGTTCCCTTTGTGAGGAA-3' and 5'-

AACCACTGAGCCAGACTCCAACAT-3') amplifies the mouse s-KL spliceform, yielding an 89 bp product (Fig. 1B). Mouse CYP24 was detected using 5'-CGTTCTGGGTGAATACACGCTAC-3' and 5'-TTCGGGTCTAAACTTGTCTCAGCATC-3' primers, and primers for mouse GAPDH were 5'-TTCCGTGTTCTACCCCCAATG-3' and 5'-TGCCTGCTTCACCACCTTCTT-3'.

Data from qrtPCR experiments were analyzed using the comparative Ct method, normalized to an endogenous reference (GAPDH). Fold effects were calculated relative to vehicle-treated control samples and expressed as $2^{-\Delta\Delta C_t}$ according to instructions in the Applied Biosystems software.

2.2 Plasmid constructs and candidate VDRE sequences

Plasmids included pSG5-hVDR expressing human VDR [11] and pRL-null (Promega Corp., Madison WI) expressing *Renilla reniformis* luciferase. Candidate VDREs were synthesized in a single copy for electrophoretic mobility shift assay (EMSA) or as a tandem repeat of two copies for cloning into the pLUC-MCS vector (Stratagene, La Jolla CA). Each oligonucleotide contained a four-base overhang [5'-agctNNN...3'] on the plus strand and [3'...NNNctag-5'] on the complementary strand for cloning into the Hind III and Bgl II sites of pLUC-MCS vector or to allow incorporation of the ^{32}P -labeled dCTP for EMSA.

The positive control for both EMSA and luciferase experiments was a rat osteocalcin (ROC) VDRE [12], with the (tandem repeat) sequence 5'-agctCACTGGGTGAATGAGGACATTACCACTGGGTGAATGAGGACATTAC-3', in which the VDRE half elements are underlined. The human or mouse elements listed below were synthesized and cloned in a similar fashion; for brevity, only a single copy of the sequences is given.

2.3 Human candidate VDRE sequences: either direct repeats with a spacer of 3 nt (DR3) or everted repeats with a spacer of 6 nt (ER6)

hKL-1: 5'-AAGAGGGAGAATTGGGGCAGCAA-3'

hKL-2: 5'-TTCATGAACTCTACGAACCATAG-3'

hKL-3: 5'-GTATTGAACTTCTTGAACCTCTG-3'

hKL-4: 5' ACTATGACCCCTCAGCGAGGGCAGGTA-3'

hKL-5: 5'-GGGTTGACCCTAGATAGGGGCAGGGC-3'

hKL-6: 5'-CCTCTGTCCCGTTTCTCCTAATG-3'

hKL-7: 5'-CGGCAGGGCAGACAGGACATCCT-3'

hKL-8: 5'-TACTGGGTTATTGAGGTGACTTA-3'

hKL-9: 5' AGAAAGGAGAGATAGGTTAGCGT-3'

hKL-10: 5'-AACCTGTCCCATTTGTCCCAGCC-3'

hKL-11: 5'-CCCCTAACCCATCTGCCCCAGAG-3'

2.4 Mouse candidate VDRE sequences: either DR3s or ER6s

mKL-1: 5'-CCCTTCTCCTCCTTCTCCTTCTT-3'

mkL-2: 5'-TTGGTTACCCTGTGTGCCCCACCC-3'
 mKL-3: 5'-TTAAAGTTCAAGAGGGACAGATG-3'
 mKL-4: 5'-GTGGTGACCTTTCTCTCCTGGAG-3'
 mKL-5: 5'-GCCCTGACCTCTGGTAAGGTCACCTC-3'
 mKL-6: 5'-AAAAGGGGCACACAGGAGAACTG-3'
 mKL-7: 5'-TCGTTGAACCGTTCTCAGGGTACCTC-3'
 mKL-8: 5'-GAAGAGGAGAGGTAGGAGAGGGG-3'
 mKL-9: 5'-TTTCTGTCCTTTAAAGGGTTCAATAA-3'
 mKL-10: 5'-CTGGAGGAGAAAGAGGACAGCAG-3'
 mKL-11: 5'-TGCATACCCTAGAGCCAGGACACGTT-3'
 mKL-12: 5'-GAGGAGGTCAGAGAGTTCACAGG-3'
 mKL-13: 5'-AACCTCACCTTCCTCTCCTATAG-3'
 mKL-14: 5'-GGCAGGGAGACAGAGGAGAGACT-3'
 mKL-15: 5'-TGTTGGGGCACACAGGTGAAAAG-3'
 mKL-16: 5'-GGTGGGGGCAGGTGGGTGAACTG-3'
 mKL-17: 5'-TGCTGGGTGATTTGGGTCAACTT-3'

2.5 Electrophoretic mobility shift assay (EMSA)

Double stranded oligonucleotides of each VDRE were labeled with [³²P]dCTP (Perkin-Elmer, Waltham MA) by Klenow fill-in and tested by EMSA [13] along with cell lysates from COS-7 cells transfected with pSG5-hVDR and pSG5-hRXRα [14].

2.6 Ability of candidate VDREs to bind VDR and activate a luciferase reporter gene

HEK-293, COS-7 and IMCD-3 were plated at 50,000-60,000 cells per well in 24-well plates and transfected with Lipofectamine Plus Reagent (Invitrogen Corp.) according to the manufacturers directions. Briefly, each well received 1 µL of Lipofectamine Reagent, 2 µL of Plus Reagent, 20 ng of pRL-null to monitor transfection efficiency, 20-100 ng of pSG5-hVDR, and 250 ng of each pLUC-MCS plasmid. After transfection, cells were treated with 10⁻⁸ M 1,25D or ethanol vehicle for 24 hrs.

HK-2 cells were transfected with Fugene HD (Roche Applied Science, Indianapolis, IN). Each well received 5 ng of pSG5-hVDR, 20 ng of pRL-null and 270 ng of the pLUC-MCS reporter to be tested along with 1 µL of Fugene transfection reagent according to the manufacturer's protocol.

Whole cell lysates were prepared and 5 µl aliquots were evaluated in a Sirius Luminometer (Pforzheim, Germany) for firefly luciferase activity and for Renilla luciferase using the Dual Luciferase Reporter kit (Promega). Results are expressed as the ratio of Firefly/Renilla relative light units × 1000.

3. Results

3.1 Klotho mRNA expression in response to 1,25D treatment

In order to detect the m-KL and the s-KL transcripts in IMCD-3 cells, two sets of primers were designed (Fig. 1B) for qrtPCR of total RNA prepared from cells treated with 10^{-8} M 1,25D for 24 hrs. Klotho transcripts were enhanced by 1,25D in IMCD-3 cells, with m-KL showing an average 2.9-fold increase and s-KL displaying an average of 4.5-fold as compared to vehicle control (Fig. 2A).

For human klotho, a single primer set was used for both the m-KL and s-KL transcripts (Fig. 1A). As shown in Fig. 2D, electrophoresis of qrtPCR products from HK-2 cells revealed a 1.6-fold increase in both transcripts which was statistically significant when averaged over a total of 23 experiments (Fig. 2B). This increase was not only 1,25D-dependent, but also required co-transfection of VDR (Fig 2B).

The distal renal tubule is reported to be the site of highest klotho expression [1]. Consequently, murine distal convoluted tubule cells (mpkDCT, a generous gift of Dr. Pawel Kiela and Fayez Ghishan, University of Arizona, Tucson [8]), were tested for induction of klotho mRNAs by 1,25D. As shown in Fig. 2C, both the m-KL and s-KL transcripts are significantly upregulated (2.9-fold and 2.8 fold, respectively) when the distal tubule cells are treated for 24 h with 10^{-8} M 1,25D.

3.2 Identification of candidate VDREs

Regulation of klotho mRNA by 1,25D is likely VDRE-mediated. Based on reports in which VDREs were found at considerable distance from the transcriptional start site of the regulated gene [15; 16; 17], VDRE search boundaries were defined based on reported sites for the CCCTC-binding factor (CTCF), which may act as an “insulator” [18]. Accordingly, approximately 160 kb of human chromosome 13 and mouse chromosome 5 (top and bottom panels of Fig. 3, respectively) were scanned *in silico* for the presence of VDRE half elements that match those of previously published VDREs (see ref [19] for a list), organized in either a DR3 or ER6 motif. Computerized searches revealed eleven candidate human VDREs (Fig. 3, top panel, arrows) and seventeen candidate mouse VDREs (Fig. 3, bottom panel, arrows) in the region of the klotho gene.

3.3 Electrophoretic Mobility Shift Assays

Double-stranded, 32 P-labeled oligonucleotides corresponding to each VDRE were assayed for VDR binding via EMSA along with a cell lysate containing human VDR (hVDR) and human RXR α (hRXR α). The rat osteocalcin VDRE (ROC [12]) served as a positive control. The results in Fig. 3 reveal shifted bands for three candidate human VDREs located at -46kb, -31kb, and +3kb (Fig. 3, middle left panel) and seven mouse elements located at -86kb, -61kb, -59kb, -35kb, -9kb, +2kb and +9kb (Fig. 3, middle right panel). Several shifted bands showed an intensity comparable to the ROC positive control. Each shifted complex was reduced by the 9A7 γ anti-VDR monoclonal antibody (α -VDR), indicating the presence of VDR [13]. For the human klotho gene, the other eight candidate VDREs (#s 1, 4, 5, 6, 7, 9, 10 and 11) did not generate a significant band on EMSA, but a VDRE reported by Wang et al. [20], GGTTCa tcc ATGTCA (denoted W in Fig. 3) formed an EMSA complex comparable to the ROC positive control (not shown). For the mouse klotho gene, the other ten candidate VDREs (#'s 1, 2, 4, 6, 8, 9, 10, 11, 13 and 14) did not generate a significant EMSA band (not shown).

3.4 Ability of VDREs to confer 1,25D responsiveness onto a luciferase reporter gene

To determine whether each VDRE could confer 1,25D- and VDR-dependent transactivation, each of the VDREs with positive EMSA results was annealed, cloned into the luciferase reporter plasmid pLuc-MCS, and cotransfected with pRL-Null into the kidney cell lines HEK-293 (Fig. 4A), HK-2 (Fig. 4B) and COS-7 (Fig. 4C). Luciferase assays of lysates from cells treated with 10^{-8} M 1,25D revealed a 1,25D-dependent increase in reporter linked to VDREs at -46kb (hKL-2; 5 to 19-fold) and -31kb (hKL-3; 2- to 12-fold). In contrast, the VDRE at +3K (hKL-8) showed a modest upregulation in HEK-293 (1.5-fold) and HK-2 (3-fold), but essentially no effect in COS-7 cells (Fig. 4, panels A, B and C, respectively). The mouse constructs were similarly evaluated in HEK-293 cells (Fig. 4D), yielding a positive result for only the element at -35kb (mKL-12; 10-fold).

4. Discussion

Potential regulation of *klotho* mRNA by 1,25D was inferred from studies reported by Tsujikawa et al. [21], who described the effect of pharmacologic and dietary vitamin D manipulations in mice on the expression of a 5.2 kb *klotho* mRNA species (representing m-KL [7]) as visualized by Northern blotting in whole kidney samples [21]. The 5.8 kb s-KL form was not evaluated by Tsujikawa et al. [21]. Herein we reinforce and extend these whole animal studies to show a) significant regulation of both *klotho* mRNA spliceforms, b) regulation by 1,25D in three kidney cell types, and c) identification of candidate VDRE sequences that may mediate this regulation in both mouse and human.

Renal *klotho* expression was initially believed to be solely in the distal tubule, but more recently *klotho* expression has been demonstrated in proximal tubule cells [6] and in the inner medullary collecting duct [22]. The present study not only reveals the expression of both *klotho* mRNA spliceforms in cell lines derived from all three of these renal cell types, but also demonstrates 1,25D regulation in all three settings.

The present time course observations differ somewhat from those of Tsujikawa et al. [21], who observed an increase in *klotho* mRNA peaking at 8 h and returning to basal levels by 24 h. In the current study, regulation by 1,25D is more prolonged, possibly due to the continuous presence of 1,25D in the cell culture systems used herein or to other factors related to the half-life of 1,25D in whole mice vs. a cell culture system.

The design of PCR primers (Fig. 1) allowed for detection not only of the mRNA encoding the full length, membrane isoform (m-KL), but also of the truncated, "secreted" form of *klotho* (s-KL); the proteolyzed forms of *klotho* were not evaluated in the current study. As illustrated in Fig. 2, the two spliceforms appear to be upregulated by 1,25D to a similar extent, except in the IMCD-3 cells, where 1,25D has slightly greater effect on the s-KL form. The exact mechanism underlying the alternative splicing of *kl* is not known, but the current results do not implicate 1,25D in regulating this process.

The physiologic significance of the full length, membrane form of *klotho* is well established [2]. However, the physiologic roles, if any, of the s-KL isoform are not clear. A *klotho* form containing both the KL1 and KL2 domains has been detected in both serum and cerebral spinal fluid (CSF), and has been interpreted as a proteolytic fragment of m-KL. The existence of a circulating *klotho* species that exactly corresponds to the peptide which would be produced from the alternatively spliced mRNA has not been reported in serum or CSF, although there is one unpublished report of such a fragment in mouse urine. It was concluded that this peptide (containing only the KL1 functional domain) was likely the result of proteolytic cleavage, but the possibility that this peptide might originate from the alternately spliced s-KL mRNA was not excluded [23].

Regardless of its origin, the klotho peptide that contains only the KL1 domain has been reported to possess intriguing properties by Abramovitz et al., [24], who reported that the s-KL cDNA produces a peptide that can act as a tumor suppressor in pancreatic cancer cells. Their data further indicate that a) klotho is normally expressed in human prostate; b) pancreatic cancers produce less klotho than normal tissue and c) the s-KL derived peptide is a more potent tumor suppressor than full-length klotho [24]. This group did not demonstrate enzymatic activity of the s-KL peptide; thus, the mechanism by which s-KL acts as a tumor suppressor is unclear. Nevertheless, the possibility that the s-KL mRNA, which we demonstrate is upregulated by 1,25D, might be producing a biologically active peptide that is secreted extracellularly (presumably into the lumen of the kidney tubule) warrants further investigation.

The present results identify two human VDRE sequences (hKL-2 and hKL-3) and a mouse sequence (mKL-12) that are able to bind VDR by EMSA and mediate 1,25D-dependent transactivation of a reporter gene. Several other elements (e.g., mKL17) were able to bind VDR but showed only weak activity in reporter gene assays. A VDRE-like element located by Wang et al. [20] at -4kb relative to the human klotho start site ("W" in Fig. 3) also showed significant binding in the gel shift assay but very weak activity in reporter gene assays (data not shown). These results therefore suggest that the human elements hKL-2 and hKL-3 and the mouse element mKL-12 are strong candidates for mediating 1,25D control of klotho expression *in vivo*, but a role for the other elements, including hKL-8 and the VDRE reported by Wang et al. [20] at -4K, cannot be ruled out at present.

Inspection of VDREs that were able to confer 1,25D responsiveness reveals that the mouse mKL-12 element is identical to the highest affinity VDRE represented by the sequence RGGTCAXxgRGTTCa [19], where R=purine and x=any base (Fig. 4E), and both human elements hKL-2 and hKL-3 differ by only one base from this "ideal" VDRE. Thus, those elements with the closest similarity to an "ideal" VDRE are also the most active in the assays utilized in the current study.

The functional importance of vitamin D responsive elements remotely located from the transcription start site has been reported with other VDR-regulated genes (e.g., [15; 16; 17]), consistent with the notion that distal VDREs are brought into proximity with the promoter by DNA looping [25]. The assumption in choosing a genomic interval for our bioinformatic VDRE search of Klotho loci was that the only boundaries for this type of interaction are those defined by CTCF binding sites [26].

The significance of 1,25D-VDR regulation of klotho for regulation of blood phosphate is very clear. Activation of 1,25D synthesis in response to low blood calcium carries with it the risk of hyperphosphatemia, since 1,25D-VDR actions promote intestinal absorption and kidney reabsorption of phosphate. Upregulation by 1,25D-VDR of FGF23 production in bone and klotho production in kidney insures that excess phosphate can be excreted to prevent hyperphosphatemia and associated problems (e.g., ectopic calcification) that are associated with aging.

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***Highlights**

- Both klotho mRNA spliceforms are regulated by 1,25-dihydroxyvitamin D (1,25D)
- Regulation occurs in cell lines from kidney proximal, distal and collecting tubules
- Candidate vitamin D response elements (VDREs) found near mouse and human *kl* genes
- Two human VDREs and one mouse VDRE are active in reporter gene assays
- Klotho regulation by 1,25D may potentiate phosphaturic effect of FGF23

A Human *Kl* gene - chr 13 **B** Mouse *kl* gene - chr 5 **Fig. 1.**

Schematic of human (A) and mouse (B) klotho splicing patterns. Exons are represented as boxes numbered 1-5; the ATG start codons and stop codons (either TAG or TAA) are also indicated. Brackets illustrate the mRNA splicing mechanism for membrane klotho (m-KL, shown above the exons) or secreted klotho (s-KL, below the exons). The resulting transcripts are shown beneath each gene with the location of PCR primers (and predicted product size).

Fig. 2.

Klotho splice forms in cell lines from different portions of the kidney tubule. qrtPCR primers (Fig. 1) were used to detect mRNA spliceforms in cell lines derived from intermedullary collecting duct (IMCD-3, panel A), distal convoluted tubule (mpkDCT, panel C) or proximal convoluted tubule (HK-2 cells, panels B and D). IMCD-3 and mpkDCT cells were treated with 1,25D (10^{-8} M) for 24 hrs prior to total RNA isolation. Human HK-2 cells were cotransfected with a VDR expression plasmid and treated with 10^{-8} M 1,25D for 24 hrs. The CYP24A1 gene was used as a positive control in all cell lines.

Fig. 3.

Candidate VDREs in the human and mouse klotho loci. Flanking regions are bracketed by CTCF-binding sites (insulators) as discussed in the text. Seventeen mouse and 11 human elements located bioinformatically are numbered relative to the transcription start site. An element reported by Wang et al. [20] (denoted “W” - open box) is also shown. Double-stranded oligonucleotides for each element (including four flanking bases on either side) were ^{32}P -labeled and subjected to EMSA. A rat osteocalcin (ROC) element served as a positive control. Each VDRE was tested without cell lysate (first lane), with lysate containing VDR and RXR α (second lane), and lysate plus a specific anti-VDR monoclonal antibody (α -VDR; third lane).

Fig. 4.

Ability of candidate VDREs to confer 1,25D responsiveness onto a luciferase reporter gene. Human (panels A-C) and mouse (panel D) candidate VDREs that bound VDR/RXR (see Fig. 3) were cloned into a pLUC-MCS reporter vector, co-transfected into the indicated cell lines along with a pSG5-hVDR cDNA expression plasmid and treated with 1,25D (10^{-8} M) for 24 hours. Cell lysate preparation and luciferase assays were performed as per the manufacturer's protocol (see Methods) and firefly luciferase values were normalized to Renilla luciferase. Results are shown as a fold effect of 1,25D. Panel E displays a comparison of those VDREs capable of conferring a response to 1,25D to the ROC positive control VDRE as well as to a consensus VDRE (R = purine base).