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Vitamin D_3 Supports Osteoclastogenesis via Functional Vitamin D Response Element of Human RANKL Gene Promoter

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Abstract Receptor activator of NF-κB ligand (RANKL) has been identified as requisite for osteoclastogenesis. To elucidate the molecular mechanism that conducts its catabolic action on bone, the effect of 1α,25 dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) on osteoclastogenesis and RANKL mRNA expression was examined by coculture, RT-PCR and nuclear run-on studies. By accelerating the transcription rate of the *RANKL* gene in SaOS2 osteoblastic cells, 1α,25$(OH)_2D_3$ enhanced in vitro osteoclast formation from peripheral monocytes. Cloning and characterization of the 5′-flanking region of the human *RANKL* gene revealed that the basic promoter comprises inverted TATA- and CAAT-boxes flanked by RUNX2 binding sites. Both electrophoresis mobility shift assay (EMSA) and transfection studies demonstrated that 1α,25$(OH)_2D_3$ activated human RANKL promoter through vitamin D responsive elements (VDRE) located at −1584/−1570 by binding VDR and RXRα heterodimers in a ligand-dependent manner. The results provide direct evidence that 1α,25$(OH)_2D_3$ augments osteoclastogenesis by transactivating the human *RANKL* gene in osteoblastic cells through VDRE. J. Cell. Biochem. 89: 771–777, 2003. © 2003 Wiley-Liss, Inc.

Key words: RANKL; osteoclasts; promoter; vitamin D_3; osteoblasts

Bone morphogenesis and remodeling are strictly regulated by the balance between bone formation and resorption [Teitelbaum, 2000; Karsenty and Wagner, 2002]. Osteoclasts, multinucleated giant cells derived from hematopoietic precursors of monocyte/macrophage lineages, play a central role in the bone resorption process. The contribution of bone marrow stromal/osteoblastic cells has been shown, by in vitro coculturing, to be requisite for differentiation of monocyte/macrophages into osteoclasts [Takahashi et al., 1988]. Stromal/osteoblastic cells express two essential molecules for osteoclastogenesis: macrophage colony-stimulating factor (M-CSF) [Hattersley et al., 1991] and receptor activator of NF-κB ligand (RANKL) [Lacey et al., 1998; Yasuda et al., 1998; Khosla, 2001]. RANKL provides signals of differentiation, activation, and maintenance of osteoclasts by binding to its receptor RANK on osteoclast precursors [Yasuda et al., 1999; Khosla, 2001]. The RANKL/RANK signaling system has been shown to play crucial roles in the development of osteolytic bone lesions such as osteoporosis [Suda et al., 1999], cancer-associated osteolytic diseases [Kitazawa and Kitazawa, 2002c] and rheumatoid arthritis [Takayanagi et al., 2000], and is therefore, regarded as a good therapeutic target. RANKL/RANK interactions also regulate T-cell/dendritic cell communication [Anderson et al., 1997], dendritic cell survival [Anderson et al., 1997; Darnay et al., 1998], and lymph node formation [Kong et al., 1999]. Also many of the bone-seeking steroid hormones and cytokines

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stimulate [O'Brien et al., 1999] or inhibit [Wei et al., 2002] osteoclastogenesis by regulating RANKL expression; of these, 1α,25 dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) is one of the key regulators of mineral homeostasis in mammals [DeLuca, 1988].

To explore the molecular mechanism of osteoclastogenesis induced by 1α,25$(OH)_2D_3$, especially the regulation of *RANKL* gene expression signifying a catabolic action on bone, we cloned and characterized the 5′-flanking region of the human *RANKL* gene.

MATERIALS AND METHODS

In Vitro Osteoclast-Like Cell Formation, RT-PCR, and Nuclear Run-On Assay

SaOS2 human osteosarcoma cells were cultured and used for in vitro studies. Human peripheral monocytic cells (10^5/cm^2), prepared as described [To et al., 1983], were co-cultured with SaOS2 cells (10^4/cm^2) for 7 days in the presence of 10 nM 1α,25$(OH)_2D_3$ in multiwell plates (Becton Dickinson, Franklin Lakes, NJ). The cells were then stained for tartrate resistant acid phosphatase (TRAP) with a commercial kit (Sigma, St. Louis, MO), and the number of osteoclast-like TRAP-positive multinucleated (more than three) cells was counted. The primers used for RT-PCR for human RANKL were GCACATCAGAGCAGAGAAAGCGATG and GTATGAGAACTTGGGATTTTGATGC. Nuclei were extracted from SaOS2 cells treated with (10^{-10} and 10^{-8} M) or without 1α,25$(OH)_2D_3$. The suspended nuclei were transcribed in vitro for 30 min at 30°C. Denatured human RANKL cDNA, human GAPDH cDNA, and vector DNA were blotted onto nylon membranes and probed with transcribed RNA.

Cloning and Sequencing of the 5′-Flanking Region of Human *RANKL* Gene

A total of 1×10^6 recombinants from the human placenta genomic library (BD Biosciences Clontech, Palo Alto, CA) was screened by plaque hybridization. A Sac I/Xho I fragment was subcloned into the pGL3-Basic vector plasmid (Promega, Madison, WI). Both strands were sequenced by the dideoxy nucleotide termination method with an ABI PRISM 310 automated sequence analyzer (Applied Biosystems, Foster City, CA). Primer extension was carried out using the end-labeled antisense oligonucleotide complimentary to the RANKL cDNA sequence as a primer. The length of the extended product was determined by sequencing the products of the human RANKL genomic fragment using the same primer.

Electrophoretic Mobility Shift Assay (EMSA)

The nuclear extract was prepared from SaOS2 cells cultured with or without 1α,25$(OH)_2D_3$ as described [Dyer and Herzog, 1995]. For the EMSA, the following double-stranded oligonucleotides spanning the putative VDRE binding sites were designed: (−1584/−1570), 5′-AGGTCAAAGACTACA-3′, (−1418/−1404), AATTCAACCTGGTCA, and (−1383/−1369), AGGTCTAAGTGTTCA. The oligonucleotides were 5′-end labeled with γ-^{32}P-ATP by T4 polynucleotide kinase (Promega). For the supershift reaction, antibodies against VDR, GR, RXRα, RXRβ, RXRγ, RARα, RARβ, and RARγ (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the binding reaction for 45 min at 4°C. The reaction products were analyzed on a 4% nondenaturing polyacrylamide gel at room temperature in 0.5× Tris-borate–EDTA buffer for 120 min.

Transient Transfection Study

Nested deletion and site-directed mutation constructs were generated either by restriction enzyme or by PCR amplification of the cloned DNA fragments. Each of the plasmid constructs and the pRL-TK vector (Promega) were cotransfected into the SaOS2 cells by the liposome mediated Effectene™ transfection reagent (QIAGEN, Valencia, CA). The cells were harvested 24 h after transfection, and luciferase activity from cell lysates was determined with a luminometer (Model ATP-3010, Advantec, Tokyo, Japan). The transfection efficiency of each construct was standardized by the activity of Renilla luciferase.

RESULTS

In Vitro Osteoclast-Like Cell Formation, RT-PCR, and Nuclear Run-On Assay

To investigate the catabolic action of 1α,25$(OH)_2D_3$ on bone, we examined whether it could promote osteoclastogenesis by increasing human *RANKL* gene expression. As shown in Figure 1, 1α,25$(OH)_2D_3$ alone or in combination with dexamethazone (Dex) supported osteoclast-like giant cell formation. These osteoclast-like giant cells formed resorption pits on

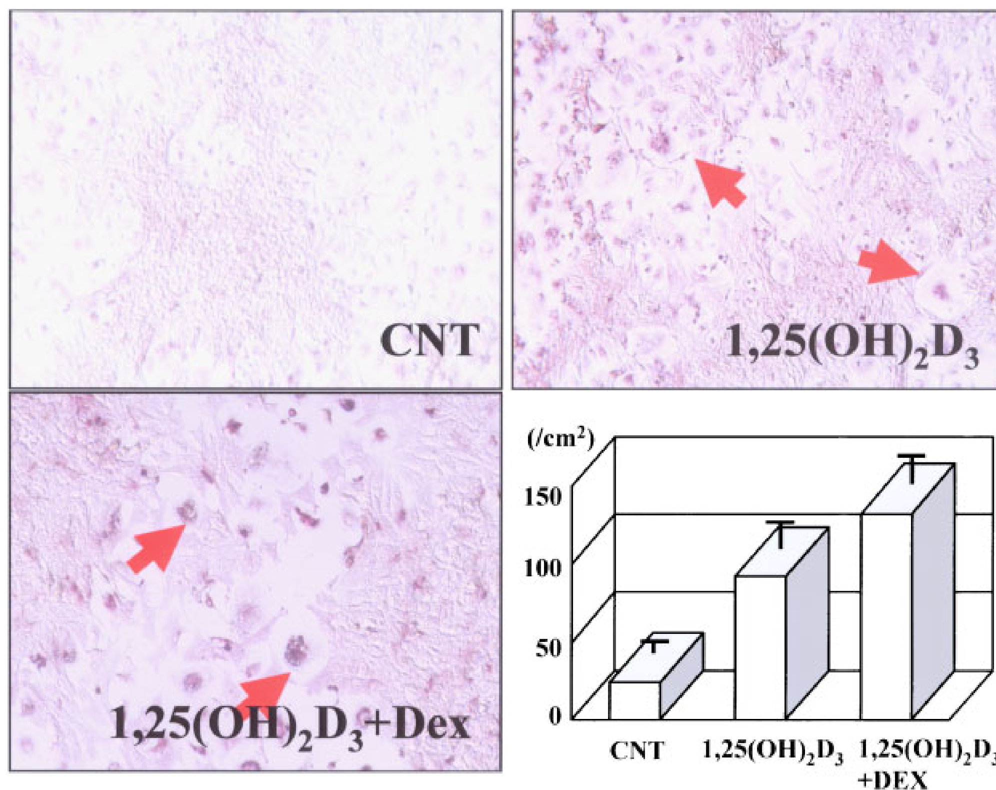


Fig. 1. In vitro effect of 1α,25 dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) on formation of multinucleated cells. Numerous tartrate resistant acid phosphatase (TRAP)-positive multinucleated cells (arrows) were formed by 1α,25$(OH)_2D_3$ alone or in combination with dexamethazone (Dex) treatment from a coculture of human osteoblastic cells, SaOS2, and peripheral blood monocytes. Bar indicates number of the TRAP-positive multinucleated cells per square centimeter (mean ± SD) of three independent assays. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

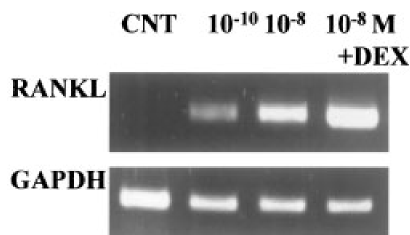
ivory slice, and 1α,25$(OH)_2D_3$ treatment increased those pits formation (data not shown). Only a few TRAP-positive osteoclast-like multinucleated giant cells were formed when 1α,25$(OH)_2D_3$ was given to monocytes alone (data not shown). Mirroring the in vitro osteoclast-like cell formation, RT-PCR, and nuclear run-on studies demonstrated that 1α,25$(OH)_2D_3$ increased the RANKL mRNA level by accelerating the transcription rate of the gene in SaOS2 cells (Fig. 2).

Cloning, Sequencing, and Mapping Transcription Start Site of the 5′-Flanking Region of Human *RANKL* Gene

We cloned 14 kb of the genomic DNA fragment where the whole 5′-untranslated portion and part of the exon 1 region of the published human RANKL [Anderson et al., 1997] were included. Figure 3 (upper panel) shows the schematic restriction enzymatic map of the cloned insert and promoter structure with putative binding sites for the transcription factors. In the lower panel, a reverse transcriptase-mediated extension of the antisense oligonucleotide primer to poly(A)+ mRNA positioned the major transcriptional start site 157 nucleotides upstream from the initial methionine site, and was assigned the +1 position. Figure 4 shows sequence data around the transcription start site and putative *cis*-acting elements of the human *RANKL* gene. The 5′-flanking sequence around the transcription initiation site of the human *RANKL* gene showed that the promoter contained inverted TATA- and CAAT-boxes at −22 and −56 bp, and a consensus binding site of RUNX2 at −201. Three candidates for VDRE were located at −1591/−1563, −1425/−1397, and −1390/−1362.

EMSA

To test whether 1α,25$(OH)_2D_3$ transactivates human *RANKL* gene by binding nuclear hormonal receptors to the VDRE, three double-stranded oligonucleotides were used as probes for EMSAs. A DNA protein band that was supershifted with anti-VDR or RXRα antibodies was observed only by EMSA with the



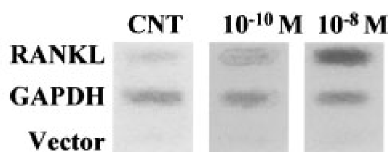


Fig. 2. Transactivation of the human receptor activator of NF-κB ligand (*RANKL*) gene by 1α,25$(OH)_2D_3$. RT-PCR (**upper panel**): mirroring the cocluture experiment, RANKL expression was upregulated by 1α,25$(OH)_2D_3$ treatment in a dose-dependent manner. Dex treatment further increased *RANKL* gene expression but kept the expression of GAPDH constant. The sizes of RANKL and GAPDH RT-PCR products were 179 and 347 base pairs (bp), respectively. Nuclear run-on analysis (**lower panel**) also showed that 1α,25$(OH)_2D_3$ accelerated *RANKL* gene mRNA transcription but kept the basic transcription rate for GAPDH constant.

−1591/−1563 DNA fragment and the nuclear extract of 1α,25$(OH)_2D_3$-treated SaOS2 cells (Fig. 5A). No specific binding or supershifted band was observed by EMSA with the −1591/−1563 DNA fragment and the nuclear extract of 1α,25$(OH)_2D_3$-untreated SaOS2 cells (Fig. 5B) or with the mutated oligonucleotide and the nuclear extract of 1α,25$(OH)_2D_3$-treated SaOS2 cells (Fig. 5C). No specific binding was observed by EMSA with either the −1425/−1397, or the −1390/−1362 probe (data not shown).

Transient Transfection Study

By transient transfection studies with promoter-reporter gene constructs, the deletion or mutation constructs lacking the intact −1591/−1563 portion of the human *RANKL* gene failed to respond to 1α,25$(OH)_2D_3$ treatment, whereas the intact construct showed a 160% increase at hour 24 (Fig. 6). These analyses showed that 1α,25$(OH)_2D_3$-dependent activation of the human *RANKL* gene is mediated through the functional VDRE located at −1584/−1570 by binding VDR and RXRα heterodimers in a ligand-dependent manner.

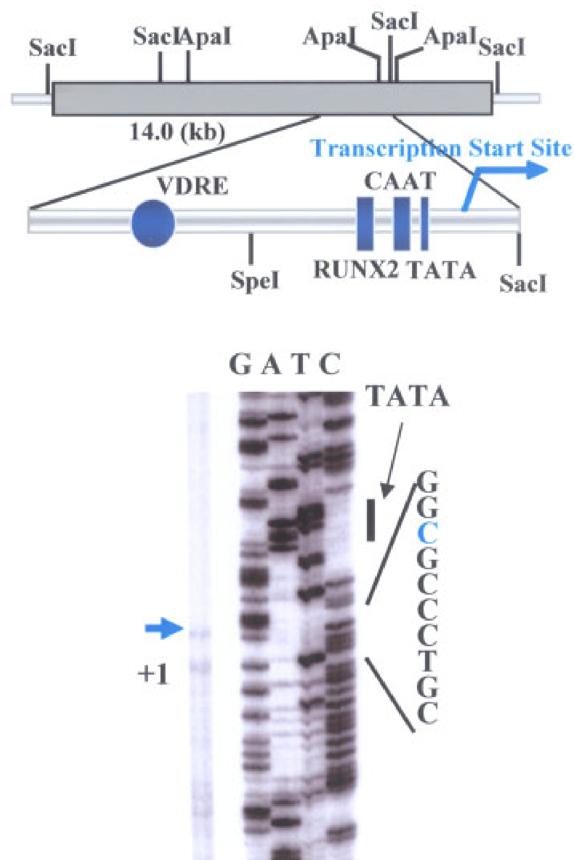


Fig. 3. Genomic structure of human *RANKL* gene and mapping the transcription start site. Restriction enzymatic map (**upper panel**) of the cloned 5′-flanking region of the human *RANKL* gene. A 2.0 kb of the Sac I–Xho I fragment was subcloned and used for the transfection studies. The product by primer extension (**lower panel**) is marked +1 (arrowhead). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

Vitamin D, one of the major regulators of calcium homeostasis, exerts its effects on the intestine, kidney, parathyroid gland, and bone [DeLuca, 1988]. It also shows a wide spectrum of effects on the hematopoietic cell linage including activation of T-lymphocyte/dendritic cell function [Griffin et al., 2001] and monocyte–macrophage differentiation [Bar-Shavit et al., 1983]. Differentiation of monocyte–macrophages into osteoclasts is, however, strictly regulated by stromal/osteoblastic cells, where the target cells of vitamin D are stromal/osteoblastic cells [Takahashi et al., 1988]. In vitro catabolic effects of vitamin D have been

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-1628  ...ataaacc tacttcccaa agttaacaaa caaaaagtgg gaagaggtca
-1578  aagactacaa ggagtagaat taacgtcaat tgtttctatg tttgagtctg
       VDRE
-1528  aaaatttttt gtcccttctc caccaaccta tatattgata cacatataaa
-1478  tgctaaaggc atttttgaat ttgaacagat cattttcttt gtatggc...
 ...   .....                                          .....
 -288  ...tttagca aaggtgtcct ctgcgtcttc cttaacccat ctcttggacc
 -238  tccagaaaga cagctgagga tggcaagggg agtctggaac cactggagta
                                                 RUNX2
 -188  gcccccagcc tcctccttgg agggcccccca tgaaggaggc ccttcagtga
 -138  cagagattga gagagaggga gggcgaaagg aaggaagggg agccagaggt
  -88  gggagtggaa gaggcagcct cgcctggggc tgattggctc ccgaggccag
                                       CCAAT-box
  -38  ggctctccaa gcggtttata agagttgggg ctgccgggcg ccctgccccgc
                     TATA-box                Transcription Start Site
  +12  tcgcccgcgc gccccaggag ccaaagccgg gctccaagtc ggcgccccac
  +62  gtcgaggctc cgccgcagcc tccggagttg gccgcagaca agaaggggag
 +112  ggagcgggag agggaggaga gctccgaagc gagagggccg agcgcca...
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Fig. 4. Human *RANKL* gene promoter sequence. Inverted TATA- and CAAT-boxes were located 22 and 56 bp upstream of the transcriptional start site. Consensus binding sites of RUNX2 were identified at −201 bp. A direct repeat of the steroid hormone responsive element spaced by three nucleotides (aggtcaaagactaca) was located at −1591/−1563. The whole sequence of the subcloned 2.0 kb fragment is registered to GenBank (accession number AF544022).

reported in both organs [Holtrop and Raisz, 1979] and cell culture systems [Takahashi et al., 1988]. We also observed that vitamin D accelerated in vitro osteoclastogenesis by upregulating *RANKL* gene expression in SaOS2 cells. Furthermore, we have, for the first time here, identified human RANKL as one of the target genes of vitamin D, and have shown that the VDR–RXRα heterodimers convey the signal by acting through the *cis*-regulatory element of the gene—a molecular mechanism of the catabolic effect of vitamin D on bone. In the mouse RANKL promoter, VDRE is also located about 1 kb upstream of the basic promoter; however, as an inexact direct repeat of the steroid response elements overlapped by a cAMP-response element-like sequence, RXRα, but not RXRβ, forms heterodimers with VDR to bind to the VDRE [Kitazawa and Kitazawa, 2002a]. Except for these differences, the basic structure of both mouse and human promoters is well preserved and composed of inverted TATA- and CAAT-boxes and a consensus binding site of RUNX2 flanked by VDRE [Kitazawa et al., 1999; Kitazawa and Kitazawa, 2002a], indicating the importance of these structures for the regulation of the gene. Human RANKL promoter-reporter gene construct (pGL3-1781) used in this study, however, did not fully represent the in vitro responsiveness of SaOS2 cells to $1\alpha,25(OH)_2D_3$ treatment. A higher chromatin structure and an epigenetic conformation [Kitazawa and Kitazawa, 2002b] or other further upstream or downstream elements may be required for the full induction of the promoter by vitamin D.

Contrasting with the in vitro catabolic effect on bone, however, administration of vitamin D metabolites stimulates osteoblastic activity [Malluche et al., 1986] and production of extracellular matrices such as type-I collagen [Owen et al., 1991], osteocalcin [Skjodt et al., 1985; Owen et al., 1991; Staal et al., 1996], and osteopontin [Owen et al., 1991; Staal et al., 1996]; at the same time, it reduces osteoclast precursors [Shibata et al., 2002], which results in an anabolic effect on bone in vivo. Although the precise mechanism is still unknown, RANKL in its GST-fused form also stimulates anabolic bone formation both in vitro and in vivo [Lam et al., 2001]. It is, therefore, possible that both the catabolic and anabolic actions of vitamin D are mediated through the common target gene, *RANKL*.

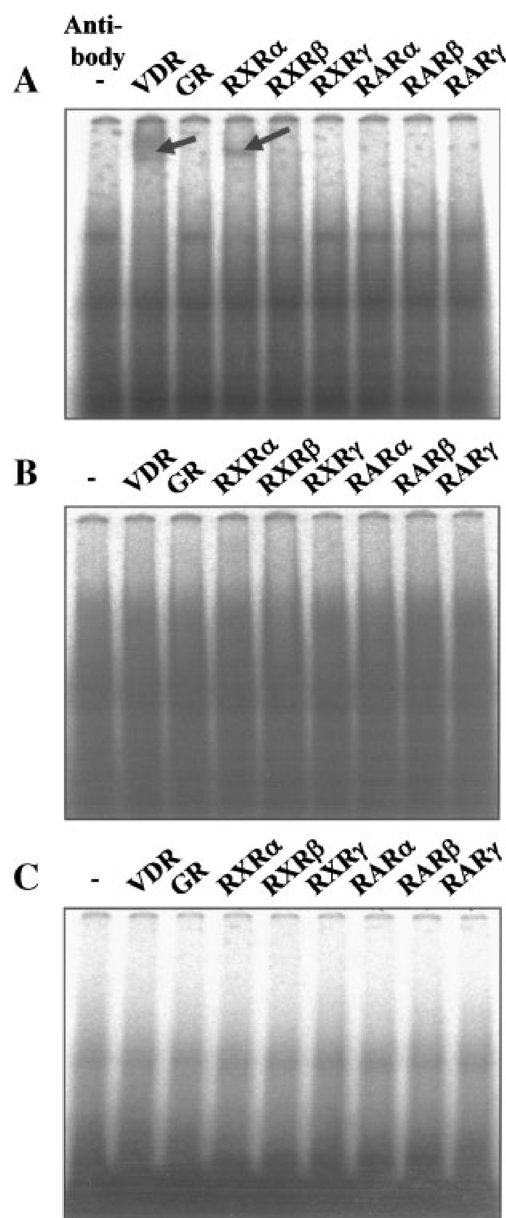

Fig. 5. The nuclear extract from SaOS2 cells cultured with (**A**) or without (**B**) 1α,25(OH)$_2$D$_3$ was subjected to the binding reaction with the ^{32}P-labeled double-stranded oligonucleotide corresponding to −1591/−1563 (aggtcaaagactaca) or mutated one (aggctcaagactctc) (**C**). The motility of the VDRE/DNA complex and the supershift complexes with anti-VDR and -RXRα antibodies (arrows) are observed exclusively in electrophoresis mobility shift assay (EMSA) with nuclear extract from 1α,25(OH)$_2$D$_3$-treated SaOS2 cells (A).

Further analysis of the human RANKL promoter will be useful in disclosing the molecular switching mechanism between the catabolic and anabolic actions of vitamin D, and in characterizing the functional interdependence between immune and bone systems in a bone marrow microenvironment.

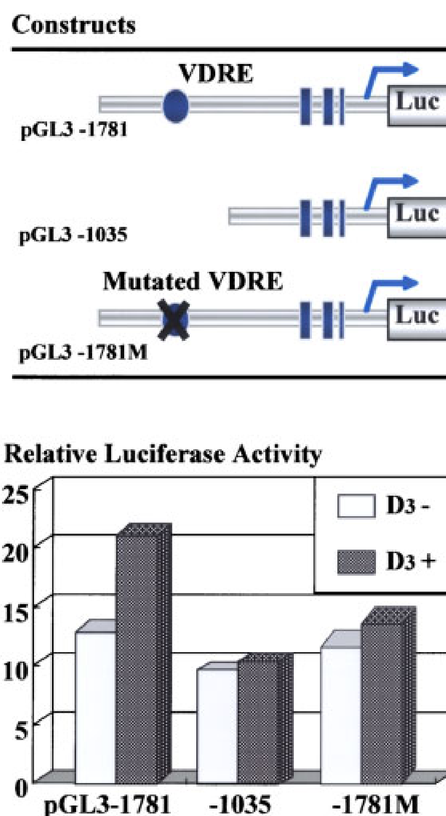

Fig. 6. Transient transfection studies using deletion or mutation constructs. 1α,25(OH)$_2$D$_3$ treatment increased RANKL promoter activity to 160% in pGL-1781, which was almost nullified in pGL-1035. Introduction of mutation (aggtcaaagactaca to aggctcaagactctc) to the VDRE site of the pGL-1781 construct also nullified the inductive effect of 1α,25(OH)$_2$D$_3$ on the RANKL promoter. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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