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Modulation of Mouse RANKL Gene Expression by Runx2 and Vitamin D_3

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ABSTRACT

The expression of receptor activator of nuclear factor-κB ligand (RANKL) is regulated by bone-seeking hormones such as PTH and 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). Runx2, a master gene for osteoblastic differentiation, also modulates osteoclastogenesis by regulating the RANKL gene. To elucidate the mechanism whereby runx2 and $1,25(OH)_2D_3$ regulate RANKL expression, we studied the function of runx2 on the chromatin structure and on the proximal binding sites using osteoblastic cell lines derived from normal (ST2) and runx2-deficient mice (RD-C6). Although the expression of RANKL in the steady-state was higher in RD-C6 than in ST2, $1,25(OH)_2D_3$-treatment of the cells increased it 20-fold in ST2 but only 1.8-fold in RD-C6. Transient transfection studies with proximal RANKL 2kb promoter, runx2 knock-down in ST2, and forced expression of runx2 in RD-C6 all confirmed that runx2 set the steady-state expression of the RANKL gene at a low level, but exerted a positive effect on enhanced transcriptional activity in response to $1,25(OH)_2D_3$. Also, assessment of the acetylation status of the area spanning 40 kb upstream of the basic promoter in ST2 and RD-C6 by ChIP assay revealed that whereas H3 and H4 histone acetylation was detected even in the steady-state in RD-C6, it was detected only with $1,25(OH)_2D_3$ in ST2. In the steady-state, runx2 may suppress RANKL gene by condensing the chromatin structure; however, it exerts a positive effect on $1,25(OH)_2D_3$-induced RANKL transcription when the proximal runx2 sites are accessible. Thus, RANKL expression in stromal/osteoblastic cells is keenly regulated by $1,25(OH)_2D_3$ which transactivates the gene at two different levels. J. Cell. Biochem. 105: 1289–1297, 2008.

KEY WORDS: RANKL; runx2; VDRE; CHROMATIN REMODELING; GENE PROMOTER

Skeletal tissue is formed through mostly endochondral and partly membranous ossification processes and is maintained through remodeling and turnover processes whereby the balance between bone formation and resorption determine bone mass. Osteoclasts are multinucleated giant cells specialized in bone resorption [Suda et al., 1999; Boyle et al., 2003; Teitelbaum and Ross, 2003]. The interaction between receptor activator of nuclear factor-κB ligand (RANKL) expressed on osteoblastic cells and its receptor RANK, expressed on osteoclast precursors, triggers osteoclastic differentiation and activation [Lacey et al., 1998; Yasuda et al., 1998]. In both physiological and pathological conditions, RANKL expression is modulated by bone-seeking hormones and cytokines through at least four different pathways: 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), cyclic AMP-protein kinase A (PKA), interleukin 6 (IL-6)/IL-11-glycoprotein 130 (gp130) and calcium ion-protein kinase C (PKC) [Suda et al., 1999; Takami et al., 2000].

Differentiation of osteoblasts from undifferentiated mesenchymal cells is regulated by runx2 as a downstream signal of bone morphogenetic protein (BMP) [Komori et al., 1997; Otto et al., 1997]. Runx2, a member of the transcription factors homologous to the *Drosophila* protein, runt [Kania et al., 1990], heterodimerizes with core binding factor β (Cbfb) to enhance DNA binding activity [Yoshida et al., 2002]. At the initial stage of osteoblastic differentiation, this heterodimer, together with Osterix, a transcription factor downstream of runx2, transduces target genes of osteoblastic phenotype such as bone sialoprotein, type I collagen and osteocalcin [Kern et al., 2001; Roca et al., 2005; Xiao et al., 2005]. At a late stage, however, runx2 suppresses the differentiation of osteoblasts [Liu et al., 2001; Geoffroy et al., 2002]. Currently

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unknown, however, is whether or not a different expression level of the RANKL gene in osteoblasts at different differentiation stages is linked to the transcriptional activity of runx2.

In our previous study, runx2 has shown a positive effect on cyclic AMP-PKA induced RANKL expression through the proximal runx2 binding sites within 400 bp from the transcription start site of the mouse gene [Mori et al., 2006]. Some contradictory findings between RANKL expression and runx2 have been reported: albeit being capable of binding to the far upstream RANKL gene promoter and possibly being linked to tissue-specific expression, runx2 per se does not exert much effect on RANKL gene activity in osteoblastic/stromal cells [O'Brien et al., 2002]; also, runx2 is not essential for $1,25(OH)_2D_3$-modulated RANKL gene expression in osteoblastic cells because high-dose $1,25(OH)_2D_3$ treatment transduces osteoclastic cells in a coculture of RD-C6 cells with normal mouse bone marrow macrophages [Notoya et al., 2004].

In this study, to clarify the role of runx2 in $1,25(OH)_2D_3$-induced RANKL expression, especially from the viewpoint of the function of runx2 in the RANKL basic promoter region and of chromatin structural changes in osteoblastic/stromal cells, we investigated the effect of runx2 on RANKL gene expression both in the steady-state and under $1,25(OH)_2D_3$-treated conditions, with the use of osteoblastic/stromal cell lines derived from normal and runx2-deficient mice.

MATERIALS AND METHODS

CELL CULTURE AND OSTEOCLAST-LIKE CELL FORMATION

Mouse bone marrow stromal cell line ST2 [Ogawa et al., 1988] (Riken, Tsukuba, Japan) and runx2−/− mouse-derived mesenchymal cell line RD-C6 [Liu et al., 2007] cultured in phenol red-free αMEM (Sigma, St Louis, MO, USA) supplemented with 2% charcoal-stripped fetal bovine serum were treated with 10^{-8} M or 10^{-7} M of $1,25(OH)_2D_3$ (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) for 12 h, and subjected to quantitative real-time reverse transcription (RT)-PCR. ST2 and RD-C6 cells ($2 \times 10^4/cm^2$) were separately co-cultured with mouse bone marrow macrophages (BMM) ($2 \times 10^5/cm^2$) isolated from the femora and tibiae of 8-week-old male mice [Kitazawa et al., 1995] for 7 days with or without 10^{-8} M or 10^{-7} M of $1,25(OH)_2D_3$ and/or 10^{-8} M of dexamethasone (Dex) (Sigma); the cells were then stained for tartrate-resistant acid phosphatase (TRACP) with a commercial kit (Sigma), and the number of osteoclast-like TRACP-positive multinucleated (>3 nuclei) cells was counted. The co-culture experiment was repeated 3 times; the representative results are expressed as the means ± SD of the number of osteoclast-like cells obtained from four culture-wells.

PLASMID CONSTRUCTS

The 5′-flanking region of the mouse RANKL gene was cloned [Kitazawa et al., 1999], and the 2kb upstream from the transcription start site was ligated into a pGL3 Basic vector (Promega, Madison, WI), resulting in a pGL3-2 k vector. Nested deletion mutants pGL3-1005 and pGL3-723 were generated as previously described [Kitazawa and Kitazawa, 2002]. Constructs with mutated runx2 binding site(s) (runx2-1: AACCTCA (−368/−362) to AACCTTG, runx2-2: AACCACT (−207/−201) to AACCTTG and runx2-3: AACCCACA (−194/−189) to AACCCTTG) or mutated VDRE (mutVDRE: TGAGGTCA (−939/−932) to TGAGGAGG) were generated as previously described [Kitazawa and Kitazawa, 2002]. Mutated PCR products were ligated into the pGL3-Basic vector, resulting in pGL3-mutrunx2-2, pGL3-mutrunx2-3, pGL3-mutrunx2-2,3, pGL3-mutrunx2-1,2,3, and pGL3-mutVDRE. These constructs were transfected into ST2 or RD-C6 cells by the liposome mediated technique, LipofectAMINE (Invitrogen, Carlsbad, CA). The phRG-TK vector (Promega) was co-transfected to standardize transfection efficiency. The transfected cells were treated with 10^{-8} M or 10^{-7} M of $1,25(OH)_2D_3$ or the vehicle for 12 h, and then luciferase activity from cell lysates was measured with a luminometer (ATP-3010; Advantec, Tokyo, Japan). The transfection study was repeated 3 times; the representative data are expressed as the means ± SD of relative luciferase activity standardized by TK promoter activity obtained from four culture-wells.

GENERATION OF runx2-EXPRESSING VECTOR AND STABLE Tet-On RD-C6 CELL LINE

Mouse runx2 cDNA derived from pKSαA1 kindly gifted by Dr. Yoshiaki Ito (Institute of Molecular Cell Biology, Singapore) [Ogawa et al., 1993] was cloned into the pTRE2hyg vector (Clontech, Palo Alto, CA) to generate doxycycline (Dox)-responsible runx2 expressing vector pTRE2hyg-runx2, as previously described [Mori et al., 2006]. Dox-responsive RD-C6 cell lines were selected by transient transfection with the pTRE2hyg-Luc vector (Clontech) and by luciferase assay. Stable pTet-On-puro transfected RD-C6 cells (C6-12IA7F) [Mori et al., 2006] were transiently transfected with the pTRE2hyg-runx2 vector using LipofectAMINE. After a 6 h interval, the cells were treated with 1 μg/ml of Dox (Sigma). Runx2 mRNA and protein expression were confirmed by RT-PCR and immunoblotting, respectively. Transfected cells cultured in DMEM supplemented with 10% FBS were treated with 10 nM of $1,25(OH)_2D_3$ for 24 h, and RNA was extracted for quantitative real-time RT-PCR.

RNA EXTRACTION, REVERSE TRANSCRIPTION (RT) AND QUANTITATIVE REAL-TIME RT-PCR

One microgram of RNA isolated from each cell was reverse transcribed to produce cDNA, which was then amplified and quantified by the ABI PRISM 7300 Real Time PCR system (Applied Biosystems, Foster City, CA) using a set of primers and probes (Assay ID; runx2, Mm00501578_m1, RANKL, Mm00441908_m1, OPG, Mm00435452_m1) (Applied Biosystems). For standardization of relative mRNA expression, rodent GAPDH primers and a probe (Assay ID; Mm99999915_g1) (Applied Biosystems) were used.

RNA INTERFERENCE EXPERIMENT

The RNA molecules (small interfering RNA (siRNA)) against runx2 or HDAC3 were synthesized by Ambion (Austin, TX), with the target mRNA sequences of runx2-sense: 5′-CGAUCUGAGAUUUGUGGG-CTT-3′, runx2-antisense: 5′-GCCCACAAAUCUCAGAUCGTT-3′, HDAC3-sense: 5′-CGACAAGGAAAGUUGAUGUTT-3′, HDAC3-antisense: 5′-ACAUCAACUUUCCUUGUCGTT-3′. ST2 cells were

transfected with the siRNA using Silencer™ siRNA Transfection Kit II (Ambion) for 48 h, then total RNA was extracted for quantitative real-time RT-PCR. As a control, non-specific siRNA (contained in the commercial kit) was transfected.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

Soluble chromatin was, according to the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY), prepared from 1.0×10^6 cells fixed with formaldehyde and precleared with salmon sperm DNA/ protein A agarose-50% slurry (Upstate), then 2 ml of the supernatant solution was incubated with anti-acetyl-histone H3, anti-acetyl-histone H4 or anti-VDR antibodies overnight at 4°C with rotation. After eluting immunoprecipitates collected with salmon sperm DNA/protein A agarose-50% slurry, DNA was recovered by phenol/chloroform extraction and ethanol precipitation for use as a template for PCR. For amplification of post-immunoprecipitated DNA, the following primers were used:

RANKL-1K-S (sense): 5′-GTTTGAGGTCAGCCTGGTTCATATAG-3′;
RANKL-1K-A (antisense): 5′-GCCTCACTGCTTAAGAAATCCTTA-TGC-3′.
RANKL-40.3K-S (sense): 5′-CTCCAGGCCTTGAGTTGAC-3′;
RANKL-40.3K-A (antisense): 5′-AGGTGATTTGATTCTGGGAAC-3′.

STATISTICAL ANALYSIS

Data are expressed as means ± SD. Statistical analyses were carried out by Student's *t*-test. The level of significance was taken to be $P < 0.05$.

RESULTS

EFFECT OF 1,25(OH)$_2$D$_3$ ON RANKL, OPG, AND runx2 mRNA EXPRESSION AND ON OSTEOCLASTOGENESIS IN ST2 AND RD-C6 CELLS

The effect of 1,25(OH)$_2$D$_3$ on RANKL, OPG, and runx2 mRNA expression was assessed by quantitative RT-PCR using RNA extracted from ST2 and RD-C6 cells. Reflecting the low level of steady-state expression, 1,25(OH)$_2$D$_3$ increased RANKL expression 16-fold in ST2 cells, but only 1.8-fold in RD-C6 cells (Fig. 1A, left). In ST2 and RD-C6 cells, 1,25(OH)$_2$D$_3$ alone decreased OPG expression to 27.9% and 84.2%, respectively, compared with the vehicle treated controls, and in combination with Dex, decreased it further to less than 15% of the controls. In ST2 cells, 1,25(OH)$_2$D$_3$ decreased runx2 mRNA to 45% of its basal expression level, while RD-C6 cells did not express runx2 (Fig. 1A, right). We then assessed the capability of these cells, in a 7-day co-culture with mouse BMM, of supporting osteoclastogenesis: reflecting the reciprocal change of

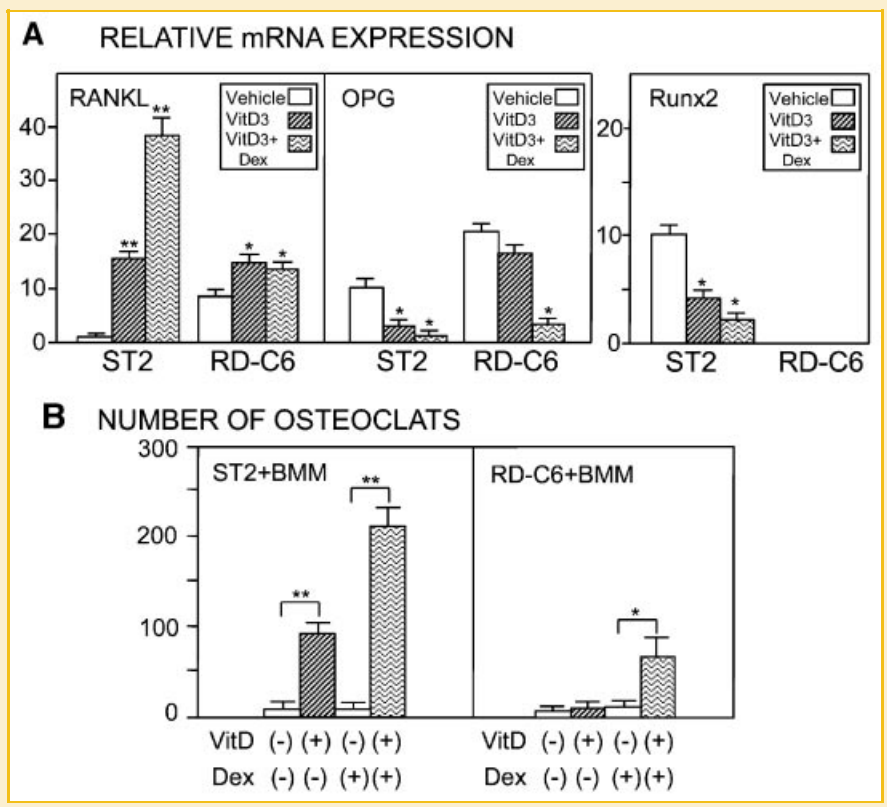

Fig. 1. Effects of vitamin D_3 on RANKL, OPG, and runx2 mRNA expression and on osteoclastogenesis in ST2 and RD-C6 cells. A: ST2 and RD-C6 cells were treated with 10^{-7}M of 1,25(OH)$_2$D$_3$ and/or 10^{-8}M of Dex for 12 h, and then total RNA was extracted for quantitative real-time RT-PCR for RANKL, OPG, and runx2. Results are expressed as the means ± SD of the relative mRNA amount standardized by GAPDH from four cultures. $^*P < 0.05$ versus vehicle, $^{**}P < 0.001$ versus vehicle. B: ST2 or RD-C6 cells were co-cultured with mouse bone marrow macrophages (BMM) for 7 days in the presence of 10^{-7} M of 1,25(OH)$_2$D$_3$ and/or 10^{-8} M of Dex, and then TRACP-positive multinucleated cells were counted. Results are expressed as the means ± SD of the number of TRACP-positive multinucleated cells from four cultures. $^*P < 0.05$, $^{**}P < 0.001$.

RANKL and OPG in ST2, $1,25(OH)_2D_3$ alone increased the number of osteoclasts 30-fold, and in combination with Dex 100-fold of the control. RD-C6 cells supported fewer osteoclasts in the presence of $1,25(OH)_2D_3$ and Dex (Fig. 1B).

THE EFFECT OF runx2 KNOCK-DOWN IN ST2 CELLS AND OF FORCED runx2 EXPRESSION IN RD-C6 CELLS ON RANKL TRANSCRIPTION

The function of runx2 in RANKL expression was confirmed through reducing the endogenous runx2 expression in ST2 cells by siRNA. Transfection with specific siRNA suppressed runx2 mRNA expression in ST2 to less than 20% of the control transfected with nonspecific (NS) siRNA (Fig. 2A, left). Mirroring the decrease in runx2 expression, the steady-state expression of RANKL mRNA increased 4.5-fold of the control. The inducible effect of $1,25(OH)_2D_3$, on the other hand, decreased 1.4-fold, while control ST2 showed a 4.8-fold increase in RANKL expression by $1,25(OH)_2D_3$ treatment (Fig. 2A, right). Conversely, runx2 expression was restored in RD-C6 cells by the Tet-On system, and RANKL mRNA expression and its promoter activity were analyzed. Forced runx2 expression with Dox treatment decreased the steady-state RANKL expression to 45% of the control and increased responsiveness to $1,25(OH)_2D_3$ from 3.5- to 4.5-fold (Fig. 2B).

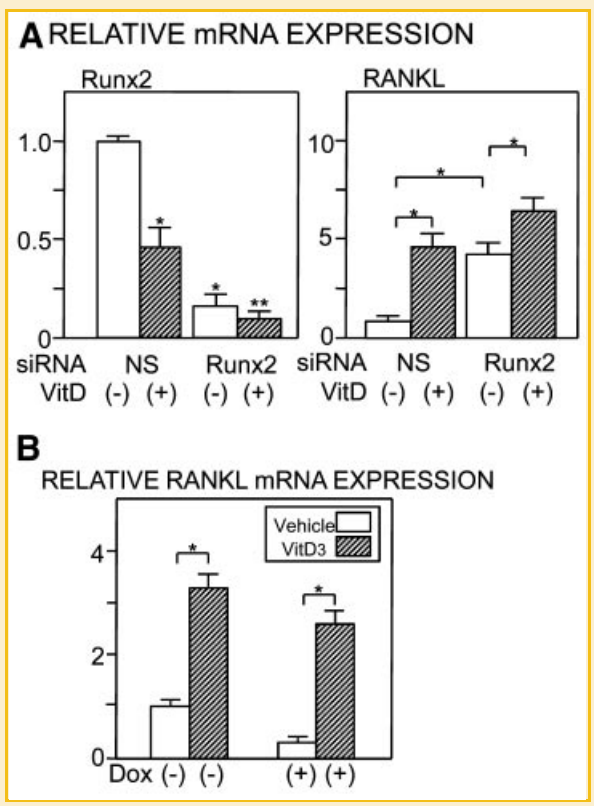

Fig. 2. The effect of runx2 knock-down in ST2 cells and that of runx2 restoration in RD-C6 cells by Tet-On system on RANKL expression. A: ST2 cells were transfected with a runx2 specific or a nonspecific (NS) siRNA and then treated with 10^{-7} M of $1,25(OH)_2D_3$ or the vehicle for 12 h before RNA extraction and RT-PCR for runx2 (left) and RANKL (right). Results are expressed as the means ± SD of the relative mRNA amount standardized by GAPDH from four cultures. $^{*}P < 0.05$, $^{**}P < 0.05$ versus NS siRNA and vehicle. B: Stably-pTet-On-puro transfected RD-C6 cells were transfected with a pTR2hyg-runx2 vector with or without 1 μg/ml of Dox, and then treated with 10^{-7} M of $1,25(OH)_2D_3$ or the vehicle for 12 h before RNA extraction and RT-PCR for RANKL. Results are expressed as the means ± SD of the relative mRNA amount standardized by GAPDH from four cultures. $^{*}P < 0.05$ versus vehicle.

HISTONE H3 AND H4 ACETYLATION STATUS AND THE EFFECT OF $1,25(OH)_2D_3$ ON HDAC3 mRNA EXPRESSION IN ST2 AND RD-C6 CELLS

Histone H3 and H4 acetylation in response to $1,25(OH)_2D_3$ was assessed by chromatin immunoprecipitation (ChIP) in ST2 and RD-C6 cells. Input and immunoprecipitated DNA with either anti-acetylated histone H3 or H4 antibodies was assessed by PCR to amplify the two regions around −40.3 and −1 kb from the transcription start site. In ST2 cells, $1,25(OH)_2D_3$ induced H3 and H4 histone acetylation in both regions, while in RD-C6 cells the acetylation was observed in both regions regardless of $1,25(OH)_2D_3$ treatment (Fig. 3A). Analyzed in ST2 and RD-C6 cells by quantitative real-time RT-PCR with or without 10^{-7}M $1,25(OH)_2D_3$, the basal HDAC3 mRNA expression in RD-C6 cells was less than half of that in ST2 cells. In ST2 cells it was suppressed by $1,25(OH)_2D_3$ treatment (Fig. 3B), mirroring the reduction of runx2 (Fig. 1A, right). Furthermore, HDAC3 inhibition by siRNA increased RANKL mRNA expression in ST2 cells at steady-state, although it did not completely reverse the inhibitory effect of runx2 (Fig. 3C).

EFFECT OF $1,25(OH)_2D_3$ ON ACTIVITY OF THE MOUSE RANKL GENE PROMOTER IN ST2 AND RD-C6 CELLS

The basic promoter region of the mouse RANKL gene contains VDRE (−937/−922) 1kb upstream from the transcriptional start site. To examine the effect of VDRE (−937/−922) deletion or mutation on promoter activity in ST2 and RD-C6 cells, constructs with deletion, pGL3-723, or mutation (TGAGGTCA to TGAGGAGG), pGL3-mVDRE, were generated (Fig. 4A). ST2 and RD-C6 cells transfected with pGL3-2K, pGL3-1005, pGL3-mVDRE and pGL3-723 were treated with $1,25(OH)_2D_3$ and subjected to luciferase assay. $1,25(OH)_2D_3$ increased the promoter activity of pGL3-2k and pGL3-1005 by 2-fold in ST2, and to 140% in RD-C6 cells. It did not affect that of pGL3-mVDRE or of pGL3-723 in either ST2 or RD-C6 cells (Fig. 4B). Thus ST2 cells showed higher activity of the proximal 2 kb promoter of the RANKL gene at the steady state and in response to $1,25(OH)_2D_3$ than did RD-C6 cells, suggesting that runx2 increased the steady-state and the $1,25(OH)_2D_3$-inducible activity of the exogenous RANKL promoter constructs. Stably-pTet-On-puro-transfected RD-C6 cells were transiently transfected with the RANKL gene promoter construct, pGL3-1005, with or without Dox. Restoration of runx2 increased the promoter activity at the steady state to 145% of the control without Dox, and it further increased responsiveness to $1,25(OH)_2D_3$ from 115% to 172% (Fig. 4C). To examine whether runx2 enhances $1,25(OH)_2D_3$/VDR binding to its responsive element (−937/−922), ChIP assay with the use of an anti-VDR antibody was done on ST2 and RD-C6 cells. In the presence of $1,25(OH)_2D_3$, a PCR product reflecting VDR binding was detected in ST2, but not in RD-C6 cells (Fig. 4D).

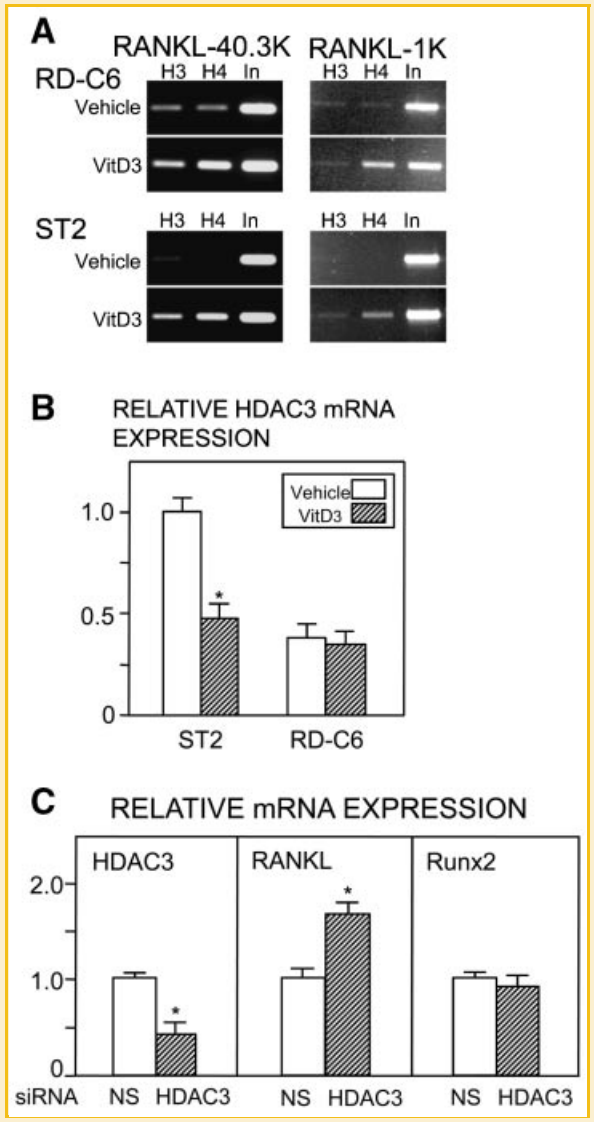

Fig. 3. Effects of $1,25(OH)_2D_3$ on chromatin condition of the regions 40 and 1 kb upstream and on HDAC3 mRNA expression in ST2 and RD-C6 cells. A: ST2 and RD-C6 cells cultured with or without 10^{-7} M of $1,25(OH)_2D_3$ were subjected to chromatin immunoprecipitation (ChIP). Input and immunoprecipitated DNA with either anti-acetylated histone H3 or H4 antibodies were assessed by PCR using sets of primers to amplify 300 bp of the regions 40 kb upstream from the transcription start site (40.3 K; left) and 270 bp containing the VDRE (−939/−932) (1 K; right). B: ST2 and RD-C6 cells were treated with 10^{-7} M of $1,25(OH)_2D_3$ for 12 h, and then total RNA was extracted for quantitative real-time RT-PCR for HDAC3 expression. Results are expressed as the means ± SD of the relative mRNA amount standardized by GAPDH from four cultures. $^*P < 0.05$ versus vehicle. C: Effect of HDAC3 silencing by siRNA on steady-state RANKL expression in ST2 cells was assessed by RT-PCR. Results are expressed as the means ± SD of the relative mRNA amount standardized by GAPDH from four cultures. $^*P < 0.05$ versus NS siRNA.

SITE-DIRECTED MUTAGENESIS STUDIES OF THE PROXIMAL runx2 SITES IN ST2 AND RD-C6 CELLS

To assess the effect of the obliteration of the proximal runx2 binding sites (−368/−362, −207/−201, and −194/−189) on RANKL gene promoter activity, 2kb mutated constructs pGL3-mR-2, -mR3, -mR-23 and -mR-123 were generated (Fig. 5A). In ST2 cells, the steady-state activity of the proximal 2 kb promoter decreased in mutated constructs pGL3-mR3, mR-23 and -mR-123. Furthermore, mutation of runx2 binding sites especially R3 mutation, nullified the inducible effect of $1,25(OH)_2D_3$ on the exogenous RANKL promoter constructs (Fig. 5B, left). On the other hand, when transfected into RD-C6 cells, these mutation constructs demonstrated no significant difference either with or without $1,25(OH)_2D_3$ treatment (Fig. 5B, right).

DISCUSSION

The endochondral ossification process is controlled by a member of the runt-homology gene family, runx2 or Cbfa1 [Komori, 2003]. During this process, hypertrophic chondrocytes are resorbed by osteo(chondro)clasts, where extensive RANKL expression by hypertrophic chondrocytes is involved in the formation and activation of osteo(chondro)clasts [Kawana and Sasaki, 2003; Takamoto et al., 2003; Kishimoto et al., 2006]. After the establishment of mature trabecular bone, while mature osteoblastic cells continuously express a low level of runx2 [Ducy et al., 1999], the steady-state expression of the RANKL gene is suppressed to a very low level [Komori, 2003]. Thus, the relation between runx2 and RANKL is somewhat complex and perplexing, albeit RANKL is one of the target genes of runx2 [Kitazawa et al., 1999, 2003; O'Brien et al., 2000, 2002].

RANKL expression is modulated by runx2 through complex mechanisms, including global changes in the chromatin structure and direct transactivation through the CRE-like site (−969/−962 bp) and proximal runx2 binding sites in the RANKL gene promoter region [Mori et al., 2006]. As confirmed by the present real-time RT-PCR, siRNA and forced expression studies, steady-state RANKL gene expression remained low in ST2 with intact runx2, but was relatively high in RD-C6 (Figs. 1A and 2A,B), indicating that runx2 exerted a net negative effect on steady-state RANKL gene expression in osteoblast/stromal cell lines. Judging from the ChIP assay (Fig. 3A), this suppression by runx2 probably requires an epigenetic mechanism through deacetylation of H3 and H4 histones that leads to chromatin condensation. Furthermore, mirroring the increase in RANKL gene expression by $1,25(OH)_2D_3$ treatment (Fig. 1A), both ST2 and RD-C6 cell lines, in the coculture with bone marrow macrophages, responded to $1,25(OH)_2D_3$ treatment and supported osteoclastogenesis (Fig. 1B). In spite of the higher level of RANKL expression in RD-C6 than in ST2 cells, the number of TRACP positive osteoclast-like cells supported by RD-C6 cells was much smaller than that supported by $1,25(OH)_2D_3$-treated ST2 cells. A relatively high level of OPG, a decoy receptor for RANKL, in RD-C6 cells even after $1,25(OH)_2D_3$ treatment (Fig. 1A) accounted for this low efficiency. On the other hand, Dex, in combination with $1,25(OH)_2D_3$, suppressed OPG expression in RD-C6 cells (Fig. 1A), confirming our previous report that Dex efficiently suppress OPG through multiple levels in a mostly runx2-independent manner [Kondo et al., 2008].

The DNA structure is conserved where runx2 and CRE are closely located [Fu et al., 2006], and since this conserved non-coding sequence is located unusually far upstream (74 kb) from the RANKL

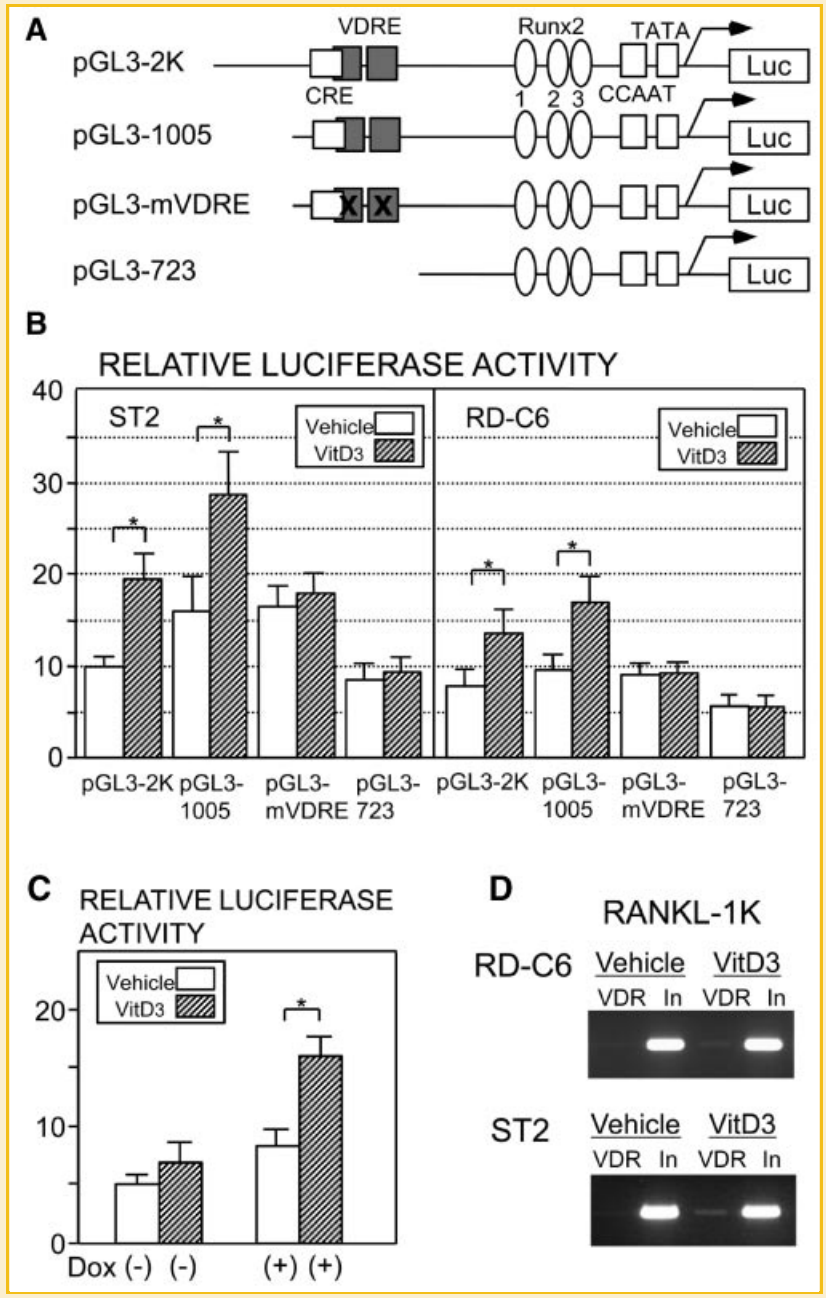

Fig. 4. Transient transfection studies using the promoter constructs of the deletion or the mutation of the VDRE of the mouse RANKL gene promoter region. A: The VDRE is located at −937/−922 in the basic promoter of mouse RANKL gene. The promoter–reporter gene constructs with deletion, pGL3-723, or mutation of the VDRE site (TGAGGTCA to TGAGGAGG), pGL3-mVDRE, were generated. B: ST2 and RD-C6 cells were transiently transfected with pGL3-2K, pGL3-1005, pGL3-mVDRE and pGL3-723, and then treated with 10^{-7} M of $1,25(OH)_2D_3$ or the vehicle for 12 h before the luciferase assay. Results are expressed as the means ± SD of the relative luciferase activity standardized by phRG-TK promoter activity obtained from four cultures. $^{*}P < 0.05$ versus vehicle. C: Stably-pTet-On-puro transfected RD-C6 cells were transfected with a pTR2hyg-runx2 vector with or without 1 μg/ml of Dox, and transfected with the mouse RANKL promoter construct, pGL3-1005, and then treated with 10^{-7} M of $1,25(OH)_2D_3$ or the vehicle for 12 h before luciferase assay. Results are expressed as the means ± SD of the relative luciferase activity standardized by phRG-TK promoter activity obtained from four cultures. $^{*}P < 0.05$ versus vehicle. D: ST2 and RD-C6 cells cultured with or without 10^{-7} M of $1,25(OH)_2D_3$ were subjected to ChIP. Input and immunoprecipitated DNA with an anti-VDR antibody were assessed by PCR using sets of primers to amplify 270 bp containing the VDRE (-939/-932) (RANKL-1K).

gene basic promoter region [Fu et al., 2006], structural alteration of global chromatin, more than direct interaction with the basic transcriptional machinery, may from a distance control RANKL gene regulation in an epigenetic manner. Indeed, the apparent contradiction between steady-state expression (Fig. 1A) and exogenously transfected promoter activity that is not suppressed by runx2 restoration (Fig. 4C), led us to assume the presence of a particular epigenetic mechanism whereby RANKL gene is modulated by runx2. We also confirmed, by ChIP assay, that the absence of runx2 resulted in the presence of acetylated histone, especially H4, associated with

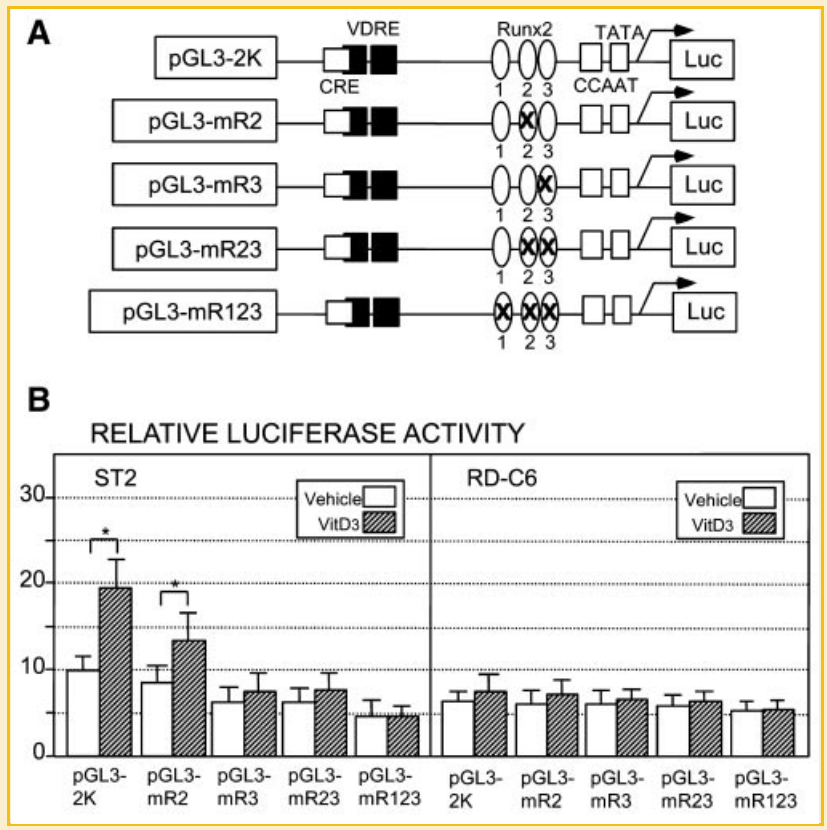

Fig. 5. Transfection studies with RANKL gene promoter constructs containing the mutation of runx2 binding sites. A: The basic promoter of mouse RANKL gene contains three putative runx2 binding sites (runx2-1: −378/−354, runx2-2: −214/−194, runx2-3: −200/−180). A 2 kb-promoter and luciferase reporter gene construct, pGL3-2K, and its mutated constructs (pGL3-mR-2, pGL3-mR3, pGL3-mR-23, pGL3-mR-123) are shown. B: ST2 (left) and RD-C6 cells (right) were transiently transfected with pGL3-2K, or its mutated constructs, pGL3-mR-2, pGL3-mR3, pGL3-mR-23 and pGL3-mR-123, and then treated with 10^{-7}M of $1,25(OH)_2D_3$ or the vehicle for 12 h before the luciferase assay. Results are expressed as the means ± SD of the relative luciferase activity standardized by phRG-TK promoter activity obtained from four cultures. $^{*}P < 0.05$ versus vehicle.

a wide-ranging area located around 40 kb upstream from the RANKL gene promoter in runx2-deficient RD-C6 cells (Fig. 3A). This remote and epigenetic mechanism through the direct recruitment of histone-modifying enzymes by runx2 is also found in many bone-related runx2-target genes, such as osteocalcin [Shen et al., 2002], $p21^{CIPI/WAF1}$ [Shen et al., 2003], and bone sialoprotein [Schroeder and Westendorf, 2005; Lamour et al., 2007].

In the RANKL gene, because the level of runx2 expression per se modulated RANKL gene expression (Fig. 2A(right) and B), and $1,25(OH)_2D_3$ treatment per se downregulated runx2 expression (Fig. 2A, left), part of the $1,25(OH)_2D_3$-induced RANKL gene activation is apparently mediated in a runx2-dependent manner. Since the decrease of runx2 by and $1,25(OH)_2D_3$ treatment alone or in combination with Dex (Fig. 1A) is comparable to that of runx2 by siRNA treatment (Fig. 2A) (which resulted in an increase in the level of RANKL expression comparable to that by and $1,25(OH)_2D_3$ treatment) this runx2-dependent mechanism may be a major pathway for $1,25(OH)_2D_3$-induced RANKL expression. Moreover, because $1,25(OH)_2D_3$ suppressed HDAC3 expression itself to half its steady-state level in ST2 cells, and this suppressive effect was lost on RD-C6 cells (Fig. 3B), the $1,25(OH)_2D_3$-induced decrease of HDAC3 expression is also activated through the runx2-dependent pathway, which may also partly contribute to RANKL gene activation. Taken together with the recent finding that multiple enhancer elements, critical for efficient RANKL gene transactivation by $1,25(OH)_2D_3$, with VDRE also span far upstream from the RANKL gene promoter to activate RANKL [Kim et al., 2007], full activation of the RANKL gene requires alteration of the chromatin structure by the sequestration of the recruited histone deacetylating enzymes during the early phase in order to loosen the chromatin structure before the direct transactivation of the RANKL gene by binding specific transcription factors to the basic promoter region. Furthermore, while HDAC3 mRNA expression in RD-C6 was not changed by $1,25(OH)_2D_3$ treatment (Fig. 3A), histone acetylation clearly increased at −40.3k and −1k. HDAC3- and runx2-independent mechanisms such as multiple enhancers located far upstream [Kim et al., 2007] may, therefore, modulate the histone acetylation status.

On the other hand, RANKL gene expression regulated by $1,25(OH)_2D_3$ treatment among runx2-deficiency, knock-down and forced expression revealed that $1,25(OH)_2D_3$ induced RANKL gene expression even in the absence or the suppressed status of runx2. The lack of responsiveness of the VDRE-null construct to and $1,25(OH)_2D_3$ treatment (Fig. 4B) indicated that this runx2-independent mechanism may be transduced mostly by a direct

binding of the VDR complex to the proximal VDRE located within 1 kb in the RANKL promoter. Interestingly, the effect of $1,25(OH)_2D_3$ treatment on the exogenously transfected RANKL gene promoter region (Fig. 4B) and on RD-C6 cells (Fig. 1A) was of almost the same level (about twofold increase). This is probably (as in the in vitro study where the exogenously transfected promoter simply mimics the status of the relaxed or activated chromatin) due to the chromatin structure around the RANKL gene promoter area being relaxed and accessible to transcription factors in RD-C6 cells. The contribution of runx2 in the runx2-independent pathway is complex and somewhat perplexing, because the lack of the runx2-binding sites slightly down-regulated exogenously transfected RANKL gene promoter activity and diminished $1,25(OH)_2D_3$-induced promoter activity. This effect was very prominent and reduced the promoter activity to nearly half its original when all three runx2 binding sites were mutated. Such reduction in promoter activity was not observed in RD-C6 cells (Fig. 5). Additionally, as in the osteocalcin gene promoter [Paredes et al., 2004], VDR-binding to VDRE (−937/−922) (as revealed by ChIP assay) was enhanced in runx2-positive ST2 cells but not in RD-C6 cells (Fig. 4D). We therefore speculate that the "appropriate" amount of runx2, namely, small enough for chromatin loosening but large enough for maintaining basic promoter activity and full induction by $1,25(OH)_2D_3$, is critical for efficient RANKL gene expression.

In conclusion, albeit Runx2 may suppress RANKL gene by condensing the chromatin structure, it exerts a positive effect on $1,25(OH)_2D_3$-induced RANKL transcription when the proximal binding sites are accessible to Runx2 by $1,25(OH)_2D_3$ treatment. Thus, RANKL expression in stromal/osteoblastic cells is strictly regulated in response to $1,25(OH)_2D_3$ which transactivates the gene at two different levels.

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