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# Epigenetic control of mouse receptor activator of NF-κB ligand gene expression

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**Abstract**

Receptor activator of NF-κB ligand (RANKL) is a membrane-bound signal transducer requisite for differentiation and maintenance of osteoclasts. RANKL expression on stromal/osteoblastic cells is tightly regulated to maintain physiological serum calcium levels and bone mass. These stromal/osteoblastic cells, however, comprise a rather heterogeneous population ranging from immature mesenchymal cells to mature osteoblasts and also respond differently to bone resorptive stimuli. In the mouse coculture system, we also have demonstrated the passage-dependent difference of cultured mouse stromal cells in supporting osteoclastogenesis due to altered RANKL gene expression. To address the issue of what molecular mechanism gives the diversity of RANKL gene expression to stromal/osteoblastic cells, we characterized the mouse RANKL gene promoter that contains two CpG clustering regions; one around the transcription start site, and the other downstream of the vitamin D response element (VDRE). Using earlier- and later-passage mouse ST2 cells, we analyzed the CpG methylation status by sodium bisulfite mapping and found that CpG loci around the transcription start site (−66/+246) were predominantly methylated in later-passage ST2 cells. Moreover, earlier- and later-passage ST2 cells transfected with a RANKL promoter construct showed the same steady-state level of luciferase activity and of the inducible effect of $1,25(OH)_2D_3$. Furthermore, the introduction of methylation to the promoter construct silenced promoter activity. The results suggest that CpG methylation around the transcription start site of the mouse RANKL gene is an important epigenetic event, and that its heterogeneity might cause the diversity of the stromal/osteoblastic cells in RANKL gene expression. 

*Keywords:* RANKL; Promoter; CpG methylation; Osteoclast; Stromal cell

Osteoclasts, derived from hematopoietic precursors of the macrophage/monocyte lineage, are multinucleated giant cells specialized to resorb bone [1,2]. Since the introduction of the mouse coculture system for in vitro osteoclastogenesis, bone marrow stromal/osteoblastic cells have been shown to play an essential role in differentiation and maintenance of osteoclast, and the stromal cell-derived osteoclast differentiation factor has been postulated [3]. As a definite osteoclast differentiation factor, a membrane-bound cytokine, receptor activator of NF-κB ligand (RANKL) has recently been identified [4,5], and most of the bone-resorbing factors such as 1,25dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$), parathyroid hormone (PTH), prostaglandin (PG) $E_2$, and interleukin (IL)-11 are thought to modulate bone resorption through RANKL gene expression on stromal/osteoblastic cells [6,7]. Therefore, in the bone marrow microenvironment, membrane-bound RANKL on stromal/osteoblastic cells is a central, if not an all-exclusive [8], factor in the regulation of osteoclastogenesis [6,7,9].

The stromal/osteoblastic cell lineage of bone tissue, on the other hand, is a heterogeneous population, ranging from immature mesenchymal cells to mature osteoblasts. Indeed, in supporting osteoclastogenesis and RANKL expression, the heterogeneity of osteoblasts and stromal cells has been demonstrated in several cell lines, including ST2, MC3T3-G2/PA6, MC3T3-E1, and NIH-3T3 [6,10]. Moreover, we have also demonstrated that ST2 cells cease supporting osteoclastogenesis with an increase in the number of culture passages [11].

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In this study, to clarify the molecular mechanism controlling RANKL gene expression, we characterized the CpG methylation status in the 5′-flanking region of the mouse RANKL gene and found that CpG methylation around the transcription start site silenced the RANKL gene.

## Materials and methods

*Cell culture and in vitro osteoclast-like cell formation.* Mouse bone marrow stromal cell line ST2 [122; Riken, Tsukuba, Japan] was cultured and maintained in αMEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma). ST2 cells were passed every 7 days; cells from a confluent culture were harvested by treatment with 0.25% trypsin/EDTA (Sigma) and subcultured at 1:8 ($10^4/cm^2$) dilution. The culture medium was changed every 3–4 days. For Northern blot, ST2 was modulated in phenol red-free αMEM supplemented with 2% charcoal-stripped fetal bovine serum and treated with 10 nM $1,25(OH)_2D_3$ and dexamethasone. Mouse bone marrow mononuclear cells ($2 \times 10^5/cm^2$), prepared as previously described [13], were cocultured with ninth- (P9) or sixteenth- (P16) passage ST2 cells ($2 \times 10^4/cm^2$) for 7 days in the presence of 10 nM $1,25(OH)_2D_3$ in multiwell plates (Becton Dickinson, Lincoln Park, NJ). The cells were then stained for tartrate resistant acid phosphatase (TRAP) with a commercial kit (Sigma), and the number of osteoclast-like TRAP-positive multinucleated (>3 nuclei) cells was counted. The coculture experiment was repeated three times and results are expressed as the mean ± SD of the number of osteoclast-like cells obtained from four culture-wells in one of three repeated experiments.

*Northern blot analysis.* A 313 bp fragment of mouse RANKL cDNA generated by RT-PCR was labeled with [α-$^{32}$P]dCTP by the random labeling method. Total RNA was extracted by RNAzol (Tel-Test, Friendswood, TX) from P9 and P16 ST2 cells cultured for 48 h with or without 10 nM $1,25(OH)_2D_3$ and dexamethasone. RNA samples (10 μg) were separated by denaturing electrophoresis in formaldehyde–agarose gels and transferred onto Hybond N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ), immobilized by UV crosslinking, prehybridized, and hybridized at 42 °C for 16 h. The membranes were washed twice in 2 × SSC containing 0.1% SDS, 1 × SSC containing 0.1% SDS, and finally in 0.1 × SSC containing 0.1% SDS at 60 °C and then visualized with BAS-EWS 4075 image analyzer (Fujix, Tokyo, Japan). Relative expression levels were estimated by comparing the optical density of the mouse RANKL and OPG mRNA bands with that of mouse GAPDH.

*Transient transfection study.* The 5′-flanking region of mouse RANKL was ligated into the promoterless and enhancerless pGL3-basic plasmid (Promega, Madison, WI). For the in vitro methylation study, the promoter–reporter gene construct was treated with *Sss*I CpG methylase (New England Biolabs, Beverly, MA, USA) at 37 °C for 1 h in the presence of *S*-adenosylmethionine supplied by the manufacturer. Each of the unmethylated and methylated plasmid constructs was transfected together with the pRL-TK vector (Promega), for standardization, into ST2 cells using Effectene Transfection Reagent (Qiagen K.K., Tokyo, Japan). Transfected ST2 cells cultured in phenol red-free αMEM supplemented with 2% charcoal-stripped fetal bovine serum were treated with $10^{-7}$ M $1,25(OH)_2D_3$, dexamethasone or the vehicle (0.1% Ethanol) 24 h prior to harvest, when luciferase activity of firefly and Renilla from cell lysates was determined with an ATP-3010 luminometer (Advantec, Tokyo, Japan). The transcription efficiency of each construct was evaluated by determining the activity of Renilla luciferase. The transfection study was repeated three times, and representative data are expressed as the mean ± SD of relative luciferase activity standardized by TK promoter activity obtained from four culture wells.

*Mapping of methylation sites by sodium bisulfite modification.* Genomic DNA was extracted and purified from ST2 cells of passage nine (P9) and sixteen (P16). The bisulfite reaction was basically carried out as previously described [14–16]. DNA (1 μg) in a volume of 50 μl TE was denatured by NaOH (final concentration, 0.2 M) for 10 min at 37 °C. Freshly prepared 30 μl of 10 mM hydroquinine, and 520 μl of 3 M sodium bisulfite, pH 5, were added to the samples. Each sample was incubated under mineral oil at 50 °C for 16 h. Modified DNA was purified with Wizard DNA purification resin according to the manufacturer's recommended protocol (Promega) and eluted with 50 μl $H_2O$. Modification was completed by NaOH (final concentration, 0.3 M) treatment for 5 min at room temperature and then by ethanol precipitation. DNA was resuspended in 20 μl $H_2O$ and used immediately or stored at −20 °C. Bisulfite-modified DNA (100 ng) was amplified by nested PCR using the following sets of converted primers covering the CpG loci downstream of VDRE (A: −920/−721) and around the transcription and translation start sites (B: −66/+357),

A-primary-sense: 5′-GAGGTTAGTTTGGTTTATATAGTAAGT TTTAG-3′;
A-primary-antisense: 5′-CAAAACTAATATCAAAATTAAACCT CACTACT-3′;
A-secondary-sense: 5′-ATAGTAAGTTTTAGGTTAGTTTAGTT TAT-3′;
A-secondary-antisense: 5′-CAACTAATAAAAACACTTACAAC TTATCTTA-3′;
B-primary-sense: 5′-AAGGAGGGTAGATGTGGGAGTGAAA GAGGTATT-3′;
B-primary-antisense: 5′-AATACAAAAACAAAACAATACAAA CCACCT-3′;
B-secondary-sense: 5′-GAGGTTGATTGGTTTTGGAGGTTAG T-3′;
B-secondary-antisense: 5′-CAATACTACAAACCACCTTTCCCA ATC-3′.

Each primer sequence was created not to contain the CpG loci of the mouse RANKL 5′-flanking region. PCR conditions were as follows: 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and a final elongation step for 5 min at 72 °C. The PCR mixture contained 1× buffer (TaKaRa) with 1.5 mM $MgCl_2$, 20 pmol of each primer, 0.2 mM dNTPs, and bisulfite-modified DNA (50 ng) in a final volume of 50 μl. Each PCR product was loaded onto a 3% agarose gel, stained with ethidium bromide, and visualized under UV illumination; gel-purified DNA was cloned into the pCR 2.1 plasmid vector (Invitrogen, Groningen, Netherlands). After transforming and culturing the competent bacteria (INVαF′; Invitrogen) overnight on an LB/agar/ampicillin plate, colonies (5–10) were randomly selected, and the recombinant plasmid was recovered for DNA sequencing with an M13F or M13R primer. The sequencing reactions for the cloned PCR products were carried out with a DNA sequencing kit (Applied Biosystems, Norwalk, CT) by the dideoxy nucleotide termination method using the aforementioned PCR conditions. The reaction products were analyzed on a 310 Genetic Analyzer (Applied Biosystems). In all molecules examined, all cytosine residues not preceding guanine residues were converted by bisulfite treatment. Bisulfite treatment, PCR, cloning, and sequencing analysis of DNA were repeated independently.

*Statistical analysis.* The significance of the data was analyzed by Student's *t* test (two-tailed).

## Results

### *Passage-dependent changes in ST2 cells supporting osteoclastogenesis and RANKL gene expression*

Cocultured with bone marrow macrophages in the presence of $1,25(OH)_2D_3$, ST2 cells of P9 supported

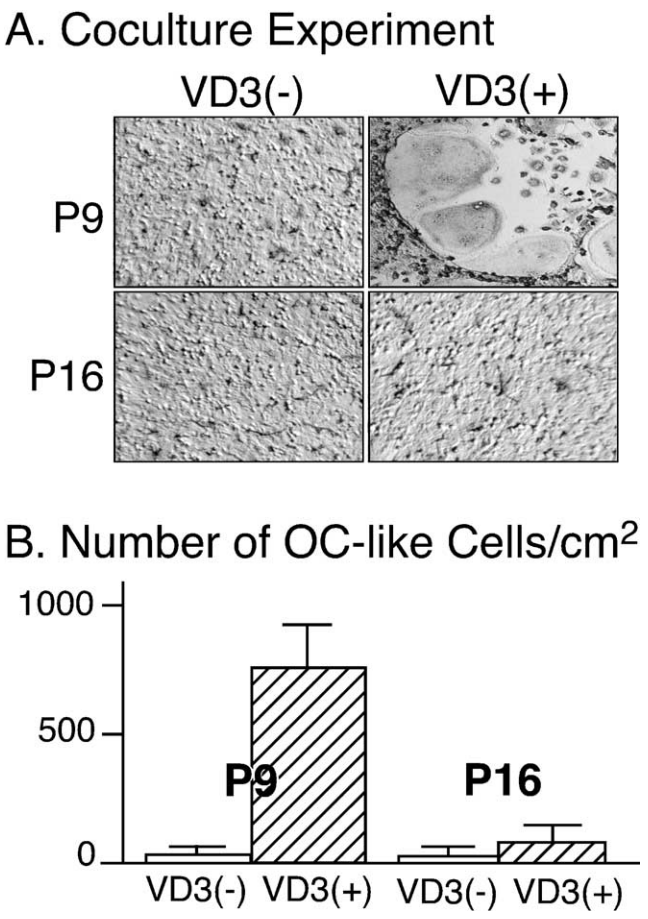


Fig. 1. Passage-dependent change in the ability of ST2 cells to promote osteoclastogenesis and RANKL gene expression analyzed by coculture and Northern blot analysis. Mouse bone marrow mononucleated cells ($10^5/cm^2$) were cocultured with P9 and P16 ST2 cells ($10^4/cm^2$) with or without 10 nM $1,25(OH)_2D_3$ for 7 days in 24-multiwell plates ($n = 4$, each), and stained for TRAP to assess osteoclast-like multinucleated cell formation. In the presence of $1,25(OH)_2D_3$, a higher number of osteoclasts were generated by P9 than by P16 cells (A, 40×). Results are expressed as the mean ± SD of four culture wells (B).

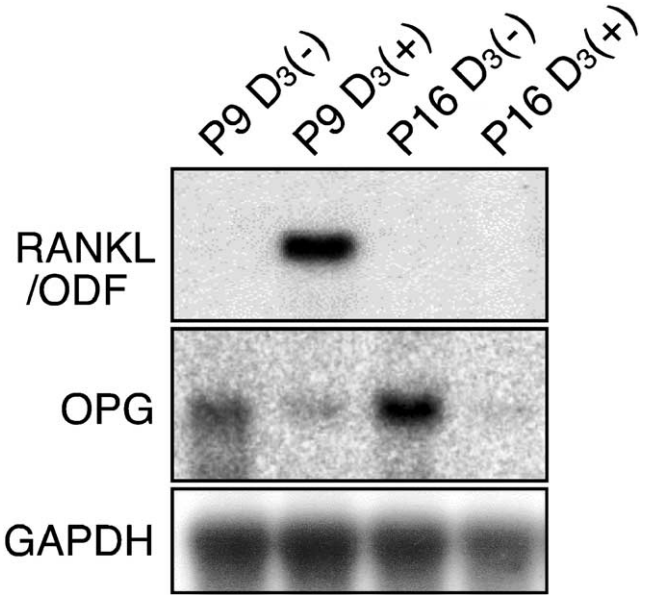


Fig. 2. Northern blot for RANKL, OPG, and GAPDH mRNA expression. Total RNA (10 μg/lane) extracted from P9 and P16 ST2 cells cultured with or without 10 nM $1,25(OH)_2D_3$ was blotted and probed with $^{32}$P-labeled mouse RANKL (upper), mouse OPG (middle) or GAPDH (lower) cDNA probes. Consistent with the osteoclast number in the coculture experiments, RANKL transcripts were detected exclusively in $1,25(OH)_2D_3$-treated P9 ST2 cells. On the other hand, OPG transcripts detected in both P9 and P16 ST2 cells were suppressed by $1,25(OH)_2D_3$ treatment.

about $800/cm^2$ of TRAP-positive multinucleated osteoclast-like cells (Fig. 1A). ST2 cells of P16, however, failed to support osteoclast-like cell formation under the same conditions (Fig. 1B). Mirroring the coculture experiment, Northern blot analysis revealed the expression of RANKL mRNA exclusively in P9 ST2 cells treated with $1,25(OH)_2D_3$; also, transcripts of OPG, a specific inhibitor for osteoclastogenesis detected in both P9 and P16 ST2 cells, were equally suppressed by $1,25(OH)_2D_3$ treatment (Fig. 2).

*Transient transfection studies*

As previously reported [11,17], the basic mouse RANKL gene promoter structure is composed of an inverted TATA-box (−28), an inverted CAAT-box (−60), and three putative Runx2 binding sites (−190, −205, and −365). One VDRE (−935) is located further upstream in the region of the mouse RANKL gene [17]. Transient transfection studies with an unmethylated construct demonstrated no significant difference in promoter activity between the ST2 cells of P9 and P16; moreover, $1,25(OH)_2D_3$ increased promoter activity 2.5-fold in both ST2 cells (Fig. 3). By contrast, in vitro methylation of the construct not only markedly reduced the promoter activity, but also abolished the inductive effect of $1,25(OH)_2D_3$ in both (Fig. 4).

*Mapping of the methylation sites of the 5′-flanking region of the mouse RANKL gene by sodium bisulfite modification*

To clarify the *cis*-regulatory mechanism of RANKL gene expression, we examined the CpG methylation status of P9 and P16 ST2 cells in two regions: (A) downstream of the VDRE, where 9 CpG loci are clustered (−920 to −800), and (B) around the transcription and translation start sites, where 39 CpG loci are clustered (−66 to +357). Sodium bisulfite-modification and high-sensitivity mapping of genomic DNA of P9 and P16 ST2 cells showed that all of the nine CpG loci were methylated in both P9 and P16 cells (Fig. 5). On the other hand, mapping of the region around the transcription and translation start sites (−66 to +357, especially, −66 to +246) revealed a larger number of methylated cytosine residues in P16 than in P9 ST2 cells

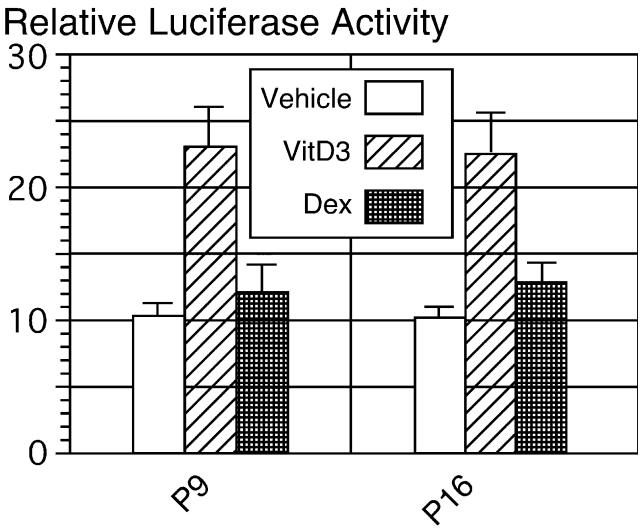


Fig. 3. Transient transfection studies with the mouse RANKL promoter construct. P9 and P16 ST2 cells were transiently transfected with RANKL promoter–luciferase reporter gene construct, together with pRL-TK, and then treated with $10^{-7}$ M $1,25(OH)_2D_3$, dexamethasone or the vehicle for 24 h prior to the luciferase assay. In both P9 and P16 cells, luciferase activity increased about 2.5- and 1.5-fold in the $1,25(OH)_2D_3$- and dexamethasone-treated cells, respectively. Vehicle-treated cells are designated as open bars. $1,25(OH)_2D_3$- and dexamethasone-treated cells are designated as lined and mesh bars, respectively. Results are expressed as the mean ± SD of the relative luciferase activity standardized by TK promoter activity obtained from four cultures.

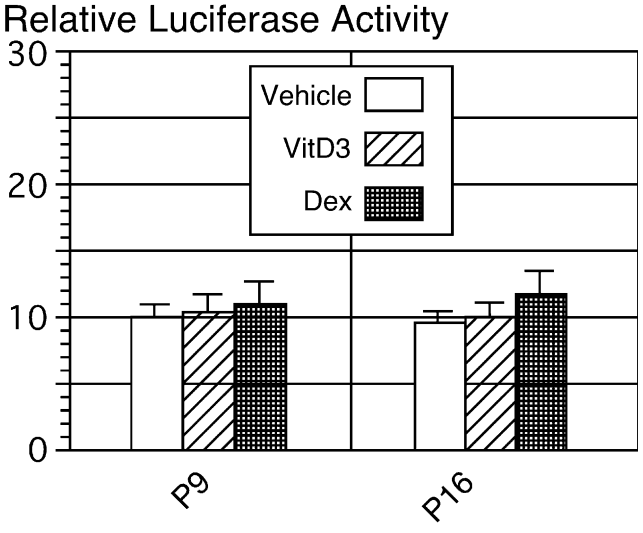


Fig. 4. Transient transfection studies with the in vitro methylated mouse RANKL promoter constract. The promoter–reporter gene construct was treated with *Sss*I CpG methylase in the presence of S-adenosylmethionine. P9 and P16 ST2 cells were transiently transfected with the construct, together with pRL-TK, and then treated with $10^{-7}$ M $1,25(OH)_2D_3$, dexamethasone or the vehicle for 24 h prior to the luciferase assay. In both P9 and P16 cells, luciferase activity was unchanged by either $1,5(OH)_2D_3$ or dexamethasone treatment. Vehicle-treated cells are designated as open bars. $1,25(OH)_2D_3$- and dexamethasone-treated cells are designated as lined and mesh bars, respectively. Results are expressed as the mean ± SD of the relative luciferase activity standardized by TK promoter activity obtained from four cultures.

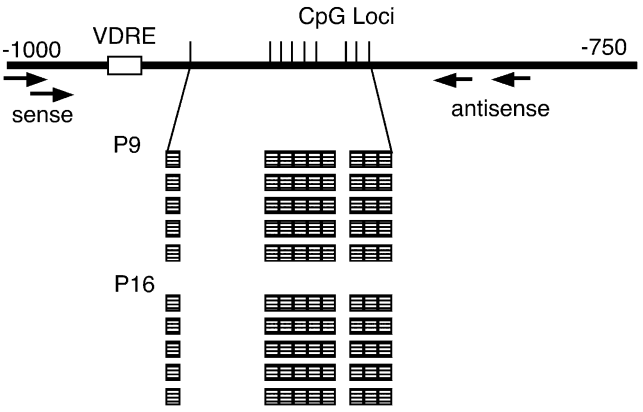


Fig. 5. Methylation status of CpG loci downstream of the VDRE in P9 and P16 ST2 cells. Genomic DNA extracted from P9 and P16 ST2 cells was subjected to sodium bisulfite-modification, PCR amplification using converted sense and antisense primers covering the nine CpG loci clustered downstream of the VDRE (−920 to − 800), cloning into a pCR 2.1 Vector, and sequencing to map the methylated cytosines. All of the nine CpG loci were methylated (patterned squares) in genomic DNA from both P9 and P16 ST2 cells.

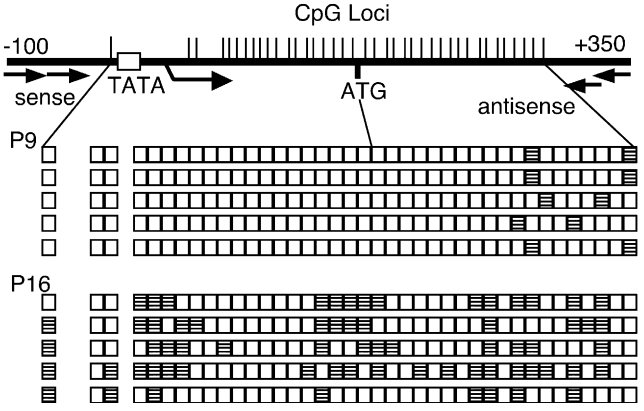


Fig. 6. Methylation status of CpG loci around the transcription and translation start sites in P9 and P16 ST2 cells. Genomic DNA extracted from P9 and P16 ST2 cells was subjected to sodium bisulfite-modification, PCR amplification using sense and antisense converted primers covering the 39 CpG loci around transcription and translation start sites of the mouse RANKL 5′-flanking region (−66 to + 357), cloning into a pCR 2.1 Vector, and sequencing to map the methylated cytosines. A larger number of methylated cytosines (patterned squares) were detected in P16 than in P9 ST2 cells.

(Fig. 6), suggesting the involvement of CpG methylation as a *cis*-regulatory mechanism around these sites.

## Discussion

There is a striking diversity and heterogeneity of osteoblasts and stromal cells in supporting osteoclastogenesis: neonatal calvarial osteoblasts strongly support osteoclastogenesis [18], whereas osteoblast maturation suppresses osteoclastogenesis, concomitant with a decrease in RANKL expression [19]. We have also

observed the passage-dependence of stromal cells in supporting osteoclastogenesis [11]. To explore the molecular mechanism providing stromal/osteoblastic cells with the versatility and specificity of expressing mouse RANKL gene, the promoter region of the gene was analyzed from the epigenetic point of view.

Northern blot analysis confirmed that the passage-dependent ability of ST2 cells to support osteoclastogenesis was attributable not to the difference of the production of OPG, a decoy receptor for RANKL [20,21], but to the silencing or unresponsiveness of the RANKL gene to bone-seeking steroid hormones. By contrast, P9 and P16 ST2 cells transiently transfected with a RANKL promoter construct containing the 1.0 kb insert showed an almost equal level of steady-state luciferase activity and responsiveness to $1,25(OH)_2D_3$ treatment (Fig. 4). This discrepancy between the two assays implies the involvement of epigenetic control of mouse RANKL gene expression. Since CpG methylation is one of the major factors controlling epigenetic regulation [22–24], we directed our attention to the CpG methylation status of the mouse RANKL gene.

The basic promoter of the mouse RANKL gene contains inverted TATA- and CAAT-boxes and three binding sites for Runx2 (also known as Cbfa-1), a transcription factor essential for skeletal development [11]. As previously reported [17], one VDRE (−935) is located further upstream in the region of the mouse RANKL gene. Furthermore, the CpG loci are clustered downstream of the VDRE and around the transcription and translation start sites (Figs. 5 and 6). Sequence-specific methylation studies showed that all CpG loci clustering downstream of the VDR were methylated in both P9 and P16 ST2 cells (Fig. 5). On the other hand, CpG loci clustering around the transcription start site (−66 to +246) were methylated mainly in later-passage ST2 cells (Fig. 6). Since RANKL expression in P9 ST2 cells with methylated CpG loci downstream of VDR was still up-regulated by $1,25(OH)_2D_3$ treatment (Fig. 3), the reason that P16 ST2 cells stopped expressing RANKL gene in the presence of $1,25(OH)_2D_3$ could be gene silencing by methylation around the transcription start site. Indeed, methylation of the 5′-untranslated and the first exonic regions is often involved in gene silencing, as described in the FMR-1 gene in the fragile X syndrome [25], the p73 gene in leukemia/lymphoma [26], the $p15^{INK4b}$ gene in lymphoma [27], and collagen pro-α 2 (I) in osteoblasts [28]. It should be noted, however, that transfection studies with in vitro methylated plasmid cannot totally represent in vivo events. Each passage of ST2 cells was, on the other hand, heterogeneous in the CpG methylation status around −66 to +357 (Fig. 6). A confluence of cultured stromal cells may induce gene- and site-specific alterations in CpG methylation upon cell passage [29]. Indeed, a recent study has demonstrated that de novo methylating activity by DNA methyltransferase (Dnmt) 3b and by an acidic Dnmt increase both in senescence and cell passage [30]. The heterogeneity and accumulation of methylation of CpG loci in the region from −66 to +246 of the RANKL gene, therefore, might account for the inability of senescent osteoblasts to support osteoclastogenesis; hence, the diversity of stromal and osteoblastic cells is achieved.

In conclusion, we characterized the mouse RANKL gene 5′-flanking region and found that expression of the RANKL gene was epigenetically regulated by cytosine methylation around the transcription start sites in mouse bone marrow stromal cells.

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