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Sp1 and Sp3 Regulate the Basal Transcription of Receptor Activator of Nuclear Factor Kappa B Ligand Gene in Osteoblasts and Bone Marrow Stromal Cells

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Abstract Receptor activator of nuclear factor kappa B ligand (RANKL), a potent regulator of osteoclast formation and function, is expressed by osteoblasts and bone marrow stromal cells. However, the molecular mechanism underlying RANKL expression in osteoblast/stromal cells remains largely unclear. Here, we characterized the molecular mechanism controlling RANKL basal transcription in osteoblast/stromal cells. We cloned a 1,103-bp murine RANKL promoter (from −953 to +150, relative to the transcription start site). Using a series of deletion mutants of the 1,103-bp promoter, we identified a 100-bp region (−154 to −54) mediating RANKL basal transcription in both osteoblasts and bone marrow stromal cells. Electrophoretic mobility shift assay (EMSA) using five overlapping oligonucleotides (Probes 1–5) spanning the 100-bp region showed that Probes 1 and 2 specifically bound nuclear proteins with high affinity from both cell types. Computer analysis revealed that Probes 1 and 2 contain a putative Sp1-binding site. Supershift assays with Sp1 and Sp3 antibodies confirmed that the nuclear proteins binding to Probes 1 and 2 are Sp1 and Sp3. Functionally, the mutation of the Sp1/Sp3 site in Probe 1 profoundly reduced the basal promoter activity while the mutation of the one in Probe 2 resulted in moderate reduction in the basal promoter activity. Moreover, the mutation of both sites abrogated the RANKL basal promoter activity, indicating that Sp1 and Sp3 play a key role in the RANKL basal transcription in osteoblasts and bone marrow stromal cells. J. Cell. Biochem. 96: 716–727, 2005.

Key words: RANKL; transcriptional regulation; transfection; promoter

Receptor activator of nuclear factor kappa B ligand (RANKL), a member of the tumor necrosis factor (TNF) superfamily, plays pivotal roles in regulating various biological processes such as bone homeostasis [Lacey et al., 1998; Yasuda et al., 1998], immune function [Anderson et al., 1997], and mammary gland development [Fata et al., 2000]. In bone, RANKL is expressed by osteoblasts and bone marrow stromal cells (referred to as stromal cells throughout this study) [Lacey et al., 1998] and mediates osteoclast differentiation and function by targeting its receptor RANK expressed on osteoclast precursors and mature osteoclasts [Hsu et al., 1999].

The unraveling of the RANKL/RANK system has helped reveal that many osteotropic hormones and cytokines regulate osteoclast formation and function through modulating RANKL expression in osteoblasts and stromal cells [Hofbauer, 1999; Hofbauer et al., 2000; Ross, 2000]. For instance, it has been known for a quite long time that in vitro generation of osteoclasts by co-culturing osteoblasts/stromal cells and osteoclast precursors requires $1\alpha,25\text{-}(OH)_2$ vitamin D_3 and dexamethasone. However, it was not clear until the discovery of the RANKL/RANK system that $1\alpha,25(OH)_2$ vitamin D_3 and dexamethasone stimulate osteoclast formation in the co-culture system by upregulating RANKL production in osteoblasts/stromal cells [Yasuda et al., 1998; Kitazawa et al., 1999]. In addition, other osteotropic hormones and cytokines such as IL-1, TNF-α, prostaglandin E2, IL-11, and parathyroid hormone (PTH) have also been shown to stimulate RANKL gene expression in osteoblasts/stromal cells [Yasuda

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et al., 1998; Hofbauer et al., 1999; Lee and Lorenzo, 1999]. In contrast, TGF-β suppresses RANKL gene expression in stromal cells [Takai et al., 1998].

While it has been established that the expression of RANKL by osteoblasts and stromal cells plays a critical role in osteoclast formation and function and also that various osteotropic factors regulate osteoclast formation and function by modulating RANKL gene expression in osteoblasts/stromal cells, the molecular mechanism by which RANKL gene expression is regulated in osteoblast/stromal cells largely remains unknown. In the present study, we characterized the molecular mechanism underlying the basal transcription of *RANKL* gene in osteoblasts and stromal cells. We found that the basal transcription of *RANKL* gene in osteoblasts and stromal cells is regulated by two *cis*-elements located close to the transcription start site (one located from −151 to −144 and the other from −124 to −117), which bind transcription factor Sp1 and Sp3. This finding will facilitate the future investigation of the molecular mechanism by which various osteotropic factors regulate the RANKL gene expression in osteoblasts and stromal cells.

MATERIALS AND METHODS

Chemicals and Reagents

Chemicals were purchased from Sigma (St. Louis, MO) unless indicated otherwise. Synthetic oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX). Antibodies against Sp1 (sc-420X) and Sp3 (sc-644X), were from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA).

Construction of a RANKL Promoter-Luciferase Reporter Plasmid and Deletion Mutants

A 1,103-bp mouse RANKL promoter (from −953 to +150 in relation to the transcription start site designated as +1) was amplified by PCR using primers derived from published sequences [Anderson et al., 1997; Kitazawa et al., 1999] and was subcloned into pGL3-basic (Promega, Madison, MI). The reporter construct in which the RANKL promoter is subcloned in sense orientation to luciferase cDNA is named as RL (−953) while the reporter plasmid in which the RANKL promoter is subcloned in anti-sense orientation to luciferase cDNA is named as RL (−953/AT). Six deletion mutants of RL (−953), RL (−753), RL (−553), RL (−403), RL (−253), RL (−154), and RL (−54) were prepared using forward primers derived from different 5′ positions (the numbers in parentheses) of the RANKL promoter and the same reverse primer as used above for amplifying the longest construct RL (−953).

Cell Cultures and Transient Transfection

Primary osteoblasts were isolated from calvarias of newborn mice as described in [Wong and Cohn, 1975]. The procedures involving mice are conducted in accord with standards of humane animal care. Isolated osteoblasts were cultured in minimal essential medium α-modification (α-MEM) containing 10% heat-inactivated fetal bovine serum (FBS). For passage, cells were lifted by treatment with collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ). Stromal cell line ST2 cells [Udagawa et al., 1989] were obtained from the RIKEN Cell Bank (Tsukuba, Japan). ST2 cells were also cultured in α-MEM containing 10% heat-inactivated FBS and were lifted by trypsinization. Both cell types were transiently transfected using LipofectAmine Plus transfection reagents from Invitrogen. One day prior to transfections, cells were plated in 6-well cell culture plates at a density of 3×10^5 cells/well. For each well, 2 μg reporter plasmid plus 0.05 μg internal-control plasmid phRL-SV40 (Promega) were used. Transfected cells were cultured for 2 days and then lysed for luciferase assays using Dual-Luciferase Reporter Assay System from Promega.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared as described by Liu et al. [2003]. Oligonucleotides were end-labeled with ^{32}P using T4 polynucleotide kinase (Invitrogen). A Probe of $2–5 \times 10^4$ cpm was incubated with 3 μg of nuclear extracts in a 20-μl volume of binding reaction (10 mM Tris-Cl, pH7.5, 100 mM NaCl, 10% glycerol, 50 ng/ml poly(dI/dC)) on ice for 20 min. For competition assays, a 100-fold excess amount of unlabeled competitors was premixed with labeled Probe before being added to the binding mixture. The binding reaction was incubated for 20 min on ice. For supershift assays, Probe was incubated with 3 μg nuclear extracts in a 20-μl volume of binding reaction for 20 min on ice, at which time 4 μg control IgG or 4 μg specific antibodies

were added, followed by incubation on ice for an additional 30 min. Reaction mixtures were resolved on 4–20% gradient gels (Invitrogen, Carlsbad, CA) using 0.5 × TBE running buffer at 4°C and 100 V for 3.5 h in a Novex Xcell II (Invitrogen) minicell electrophoresis system. The gels were transferred to 3 M blotting paper, dried, and exposed to films.

Site-Directed Mutagenesis

Point mutations were introduced in the context of the longest RANKL promoter construct, RL (−953) using a QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotides used to mutate the two Sp1/Sp3-binding sites were purified by polyacryamide gel electrophoresis. PCRs were performed in a 50-μl volume with Pfu polymerase, 10 ng of DNA template RL (−953), and 125 ng of each oligonucleotide using the following conditions: 95°C for 30 s, 1 cycle; 95°C for 30 s, 55°C for 1 min and 68°C for 12 min, 18 cycles; and 4°C park. The PCR products were treated with Dpn I (10 U) for 60 min at 37°C. XL1-blue supercompetent cells were transformed with the Dpn I-treated PCR mixture, as described in the instruction manual, and plated on ampicillin plates. Plasmids were prepared from individual colonies and sequenced to confirm the correctness of introduced mutations.

Sequence Analysis

Sequence analysis was performed using the Genetic Computer Group (Madison, WI) sequence analysis software.

RESULTS

A 1,103-bp Mouse RANKL Promoter Exhibits High Transcription Activity in Both Primary Osteoblasts and Stromal Cells

A 959-bp mouse promoter (from −954 to +5, relative to the transcription start site) was previously cloned and reported [Kitazawa et al., 1999] and the sequence of a 2,344-bp mouse genomic fragment containing the RANKL promoter (from −2,344 to −1) was deposited in GenBank (AF332141). In addition, a mouse RANKL cDNA with a 130-bp 5′ untranslated region (5′UTR) was also reported [Anderson et al., 1997]. Given that the region between the transcription start site and the translation start site may contain functional sites mediating gene transcription [Feng et al., 2000], we decided to include this region in the promoter construct to be used for this study. Thus, we designed a forward primer corresponding to the region (−954 to −924) of the reported RANKL promoter [Kitazawa et al., 1999] and a reverse primer complementary to a 5′UTR sequence 10-bp upstream of translation start site (ATG) in the reported mouse RANKL cDNA [Anderson et al., 1997] (Fig. 1A). Using these primers, we amplified a 1,103-bp mouse genomic fragment containing promoter region from −953 to +150 by PCR (Fig. 1A). Notably, the sequencing of the fragment revealed a 30-bp sequence (+6 to +35), which has not been previously reported (the underlined sequence in Fig. 1A). To ensure that our sequence is not altered by the PCR-based amplifications, we sequenced and compared two clones from two independent PCR amplifications. Interestingly, we found that our sequence from −953 to −1 is identical to the one deposited in GenBank (AF332141), but it is different from the reported sequence [Kitazawa et al., 1999] by missing one nucleotide around −253 (Fig. 1A). The difference is likely to result from variations in mouse strains and/or individual animals. The RANKL promoter was cloned into pGL3-basic either in sense orientation to luciferase cDNA, resulting in RL (−953), or in anti-sense orientation to luciferase cDNA, giving rise to RL (−953/AT) (Fig. 1B).

To examine whether the RANKL promoter is transcriptionally active in osteoblasts and stromal cells, we performed transfection assays with RL (−953) and RL (−953/AT) (Fig. 2). For these assays, we used primary osteoblasts isolated, as described by Wong and Cohn [1975]. However, since primary bone marrow stromal cells were not transfectable (data not shown), we turned to use a bone marrow stromal cell line ST2 cells [Udagawa et al., 1989]. In addition, these two cell types were also transfected with pGL3-promoter in which luciferase reporter gene is driven by a viral promoter, SV40 as positive control, and with promoterless construct, pGL3-basic as negative control (Fig. 2). The transfection assays demonstrated that the RANKL promoter exhibited high promoter activity in both primary osteoblasts (Fig. 2A) and ST2 cells (Fig. 2B). As negative control, RL (−953/AT) and pGL3-basic failed to show any promoter activity. These data demonstrated that the cloned RANKL promoter is transcriptionally active in both osteoblasts and ST2 cells.

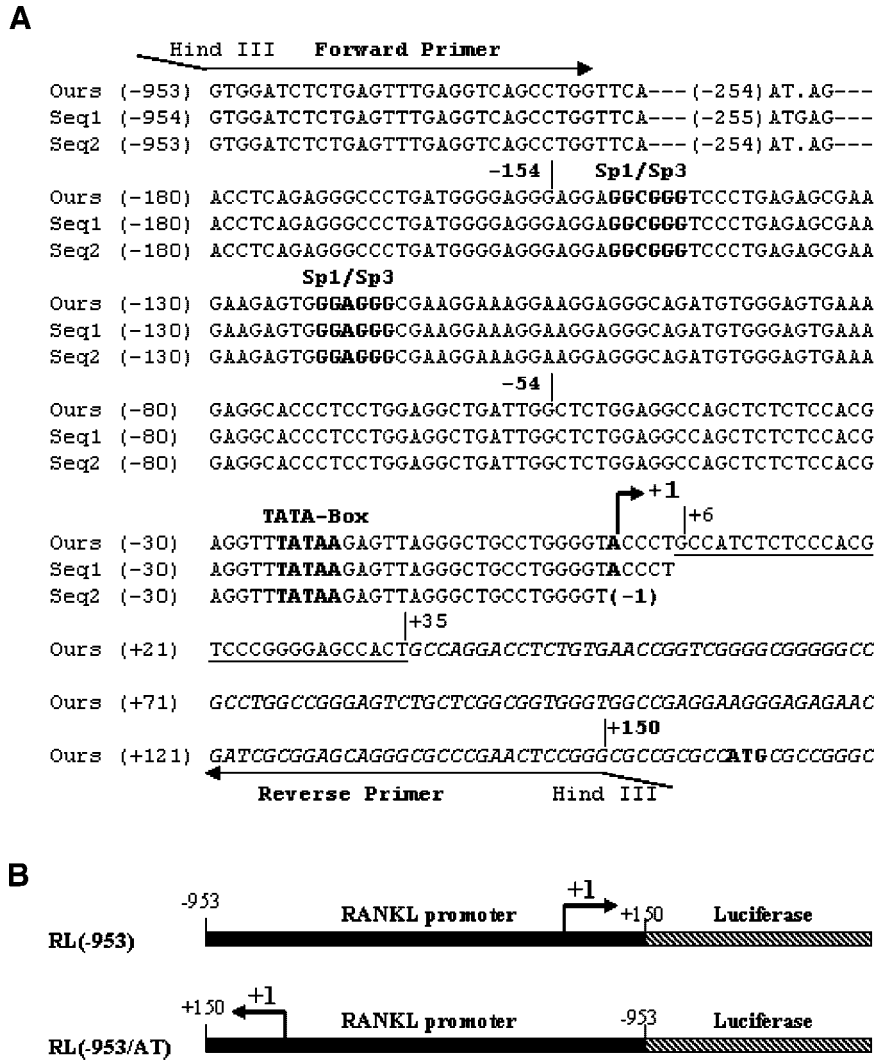

Fig. 1. Construction of mouse RANKL promoter reporter plasmids. **A**: Comparison of our RANKL promoter sequence (Ours) with one previously published (Seq1)[Kitazawa et al., 1999] and one available in GenBank (Seq2; AF332141). Our TRAP promoter was amplified by PCR using the forward and reverse primers. The two Sp1/Sp3-binding sequences mediating RANKL basal transcription and TATA-box are in bold. The sequence corresponding to the 5′UTR of the reported RANKL cDNA is in italic. The translation start site ATG is in bold. **B**: Construction of reporter constructs RL (−953) and RL (−953/AT).

Localization of a 100-bp Promoter Region Mediating the Basal Transcription of RANKL Gene in Osteoblasts and Stromal Cells

To locate a promoter region mediating the basal transcription of *RANKL* gene, we prepared six nested deletion mutants of RL (−954): RL (−753), RL (−553), RL (−403), RL (−253), RL (−154), and RL (−54) (Fig. 3A). These deletion mutants were transiently transfected into primary osteoblasts (Fig. 3B) and ST2 cells (Fig. 3C). As shown in Figure 3B, RL (−953), RL (−753), RL (−553), RL (−403), RL (−253), and RL (−154) all demonstrated considerably high promoter activities in transfected primary osteoblasts. However, RL (−54), in which an additional 100-bp region from −154 to −54 was deleted, showed a dramatic decrease in promoter activity in transfected primary osteoblasts, indicating that the 100-bp promoter region is essential for the basal transcription of *RANKL* gene in osteoblasts. Similarly, our transfection assays also demonstrated that the same 100-bp region (from −154 to 54) also regulates the basal transcription of *RANKL* gene in ST2 cells (Fig. 3C).

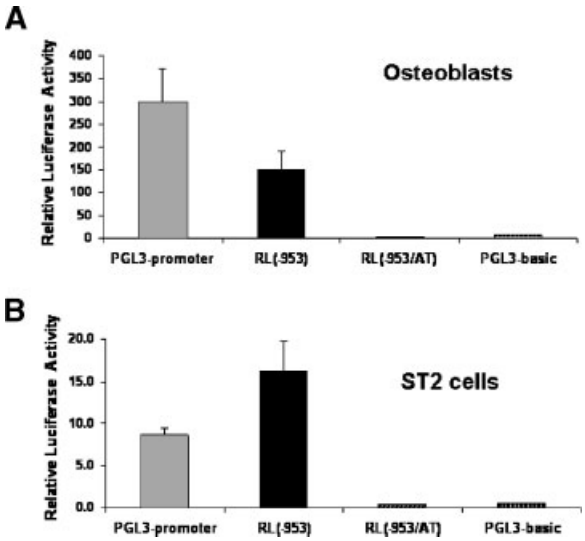

Fig. 2. The RANKL promoter is transcriptionally active in primary osteoblasts and stromal cell line ST2 cells. **A**: Primary osteoblasts were transiently transfected with pGL3-promoter RL (−953), RL (−953/AT), or pGL3-Basic, as described in Materials and Methods. The experiment was repeated twice and one result is shown. Each bar is the mean of three replicates ± SD. **B**: ST2 cells were transiently transfected with pGL3-promoter, RL (−953), RL (−953/AT), or pGL3-basic as described in (A).

Two of the Five Overlapping Oligonucleotides Spanning the 100-bp RANKL Promoter Bind Nuclear Proteins With High Affinity From Both Osteoblasts and ST2 Cells

To elucidate the molecular mechanism controlling *RANKL* gene basal transcription in osteoblasts and stromal cells, we synthesized five overlapping oligonucleotides (Probes 1–5) spanning the 100-bp RANKL promoter region (Fig. 4A). We labeled these oligonucleotides and used them as Probes to perform EMSA with nuclear extracts from either osteoblasts (Fig. 4B) or stromal cell line ST2 cells (Fig. 4C). The data indicated that Probes 1 and 2 bind nuclear proteins from both osteoblasts and ST2 cells with high affinity (Lanes 1 and 2, Fig. 4B,C). Probe 3 binds nuclear proteins from these two cell types with lower affinity (Lane 3, Fig. 4B,C). Subsequent competition showed that a considerable portion of the bands seen with Probe 3 resulted from non-specific nuclear protein binding (data not shown). Probes 4 and 5 not only gave rise to very weak bands (Lanes 4 and 5, Fig. 4B,C) but also subsequent competition indicated that the bands seen with Probes 4 and 5 mainly resulted from non-specific nuclear protein binding (data not shown). Based on these observations, we decided to focus only on the potential functional sequences present in Probes 1 and 2 in the current investigation. As shown in Figure 4B,C, both Probes 1 and 2 gave rise to three major bands (bands a–c). However, while bands a and b stably formed in all our EMSA experiments, band c is not stable. For our EMSA, we used pre-cast gels from Invitrogen. We found that the formation of band c in EMSA largely depends on the batch of pre-cast gels used. For instance, band c was almost undetectable in Fig. 5B (Lane 1) but it was very strong in Fig. 6B (Lane 1). Given these considerations, we chose to focus on the bands a and b in the present study.

Identification of Two Sp1/Sp3-Binding Sites in Probes 1 and 2 by EMSA

Next, we determined whether Probes 1 and 2 bind the nuclear proteins from osteoblasts in a specific manner. We performed competition assays with 100× excess unlabeled Probes 1 or 2 and the result demonstrated the nuclear protein binding is specific for both Probes 1 and 2 (Lane 2, Fig. 5A,B). Computer analysis suggests that both Probes 1 and 2 contain a putative binding site for the transcription factors of the specificity protein 1-like/Krüppel-like factors (Sp1-like/KLF) family [Bouwman and Philipsen, 2002; Kaczynski et al., 2003]. Thus, we also performed competition assays with 100× excess unlabeled oligonucleotide containing Sp1 consensus sequence or corresponding oligonucleotide with mutations in the Sp1 consensus sequence (Lanes 3 and 4, Fig. 5A,B). While the oligonucleotide containing Sp1 consensus sequence completely competed off bands a and b, the corresponding oligonucleotide with mutations in the Sp1 consensus sequence failed to do so, suggesting that the Probes 1 and 2-bound nuclear proteins corresponding to bands a and b belong to the Sp1-like/KLF family. Subsequently, supershift assays with antibodies against Sp1 and Sp3, two widely known members of the Sp1-like/KLF family, confirmed that the nuclear proteins binding to Probes 1 and 2 are indeed Sp1 and Sp3 (Lanes 7 and 8, Fig. 5A,B). More specifically, while "band a" consisted of both Sp1 and Sp3, "band b" resulted from binding Sp3 only (Lanes 7 and 8, Fig. 5A,B).

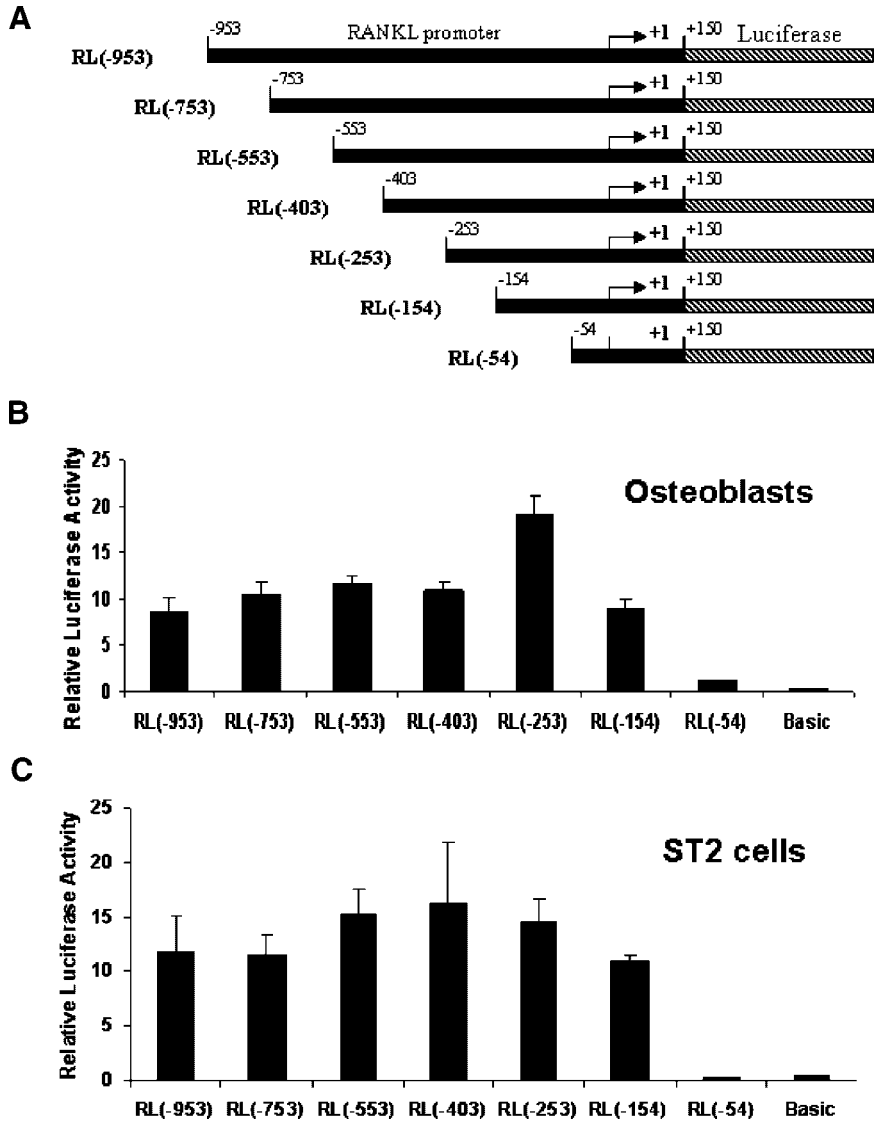

Fig. 3. Localization of a 100-bp RANKL promoter region (−154 to −54) mediating RANKL basal transcription in osteoblasts and ST2 cells. **A**: Schematic diagrams of six deletion mutants: RL (−753), RL (−553), RL (−403), RL (−253), RL (−154), and RL (−54). **B**: Transfection assays with the mutants using osteoblasts. The assay was repeated three times and a representative result is shown. Each bar is the mean of three replicates ± SD. **C**: Transfection assays with the mutants using ST2 cells. Basic: pGL3-basic.

To examine whether Probes 1 and 2 also bind Sp1 and Sp3 from ST2 cells, we repeated the same set of assays in Figure 5 with ST2 cell nuclear extracts. The data demonstrated not only that Probes 1 and 2 bind specifically the nuclear proteins from ST2 cells (Lane 2, Fig. 6A,B) but also that the ST2 cell nuclear proteins bind to Probes 1 and 2 are Sp1 and Sp3 (Lanes 3, 4, 7, and 8, Fig. 6A,B). Once again, band a in EMSA with ST2 cell nuclear extracts consisted of both Sp1 and Sp3 while band b resulted from binding Sp3 only (Lanes 7 and 8, Fig. 6A,B).

Two Sp1/Sp3-binding Sequences Are Functionally Involved in the RANKL Basal Transcription in Both Osteoblasts and ST2 Cells

Finally we determined whether two Sp1/Sp3 binding sequences are functionally involved in the RANKL basal transcription in osteoblasts and ST2 cells. To this end, we need to identify mutations in the Sp1/Sp3 binding sites that are capable of blocking the Sp1/Sp3 binding. We synthesized a mutant Probe 1 (mProbe 1), in which 4 Gs in the core binding AGGCGGGT were converted to 4 Ts, and a mutant Probe 2

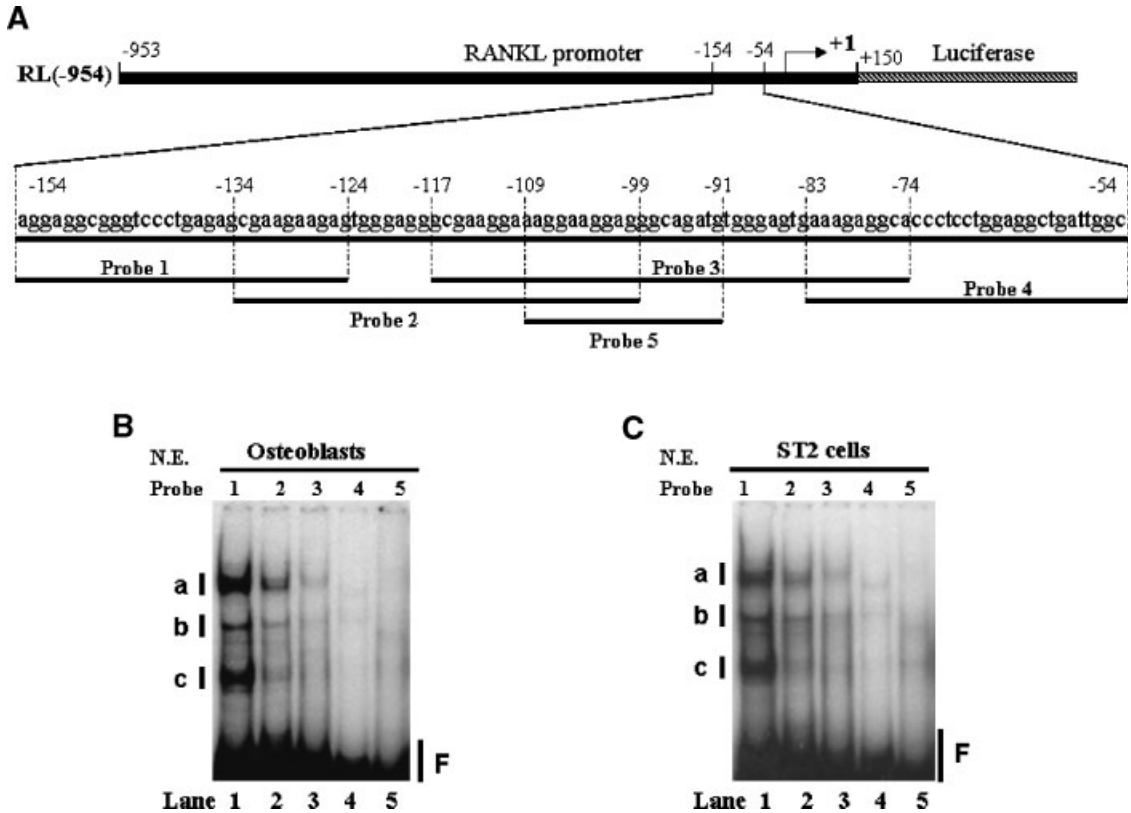

Fig. 4. Two of the five overlapping oligonucleotides spanning the 100-bp RANKL promoter bind nuclear proteins with high affinity from both osteoblasts and ST2 cells. **A**: Diagram of five overlapping oligonucleotides (Probes 1–5). **B**: EMSA with the Probes 1–5 using nuclear extracts from osteoblasts, as described in Materials and Methods. **C:** EMSA with Probes 1–5 using nuclear extracts from ST2 cells. Three bands are indicated by letters a–c. F, free Probe.

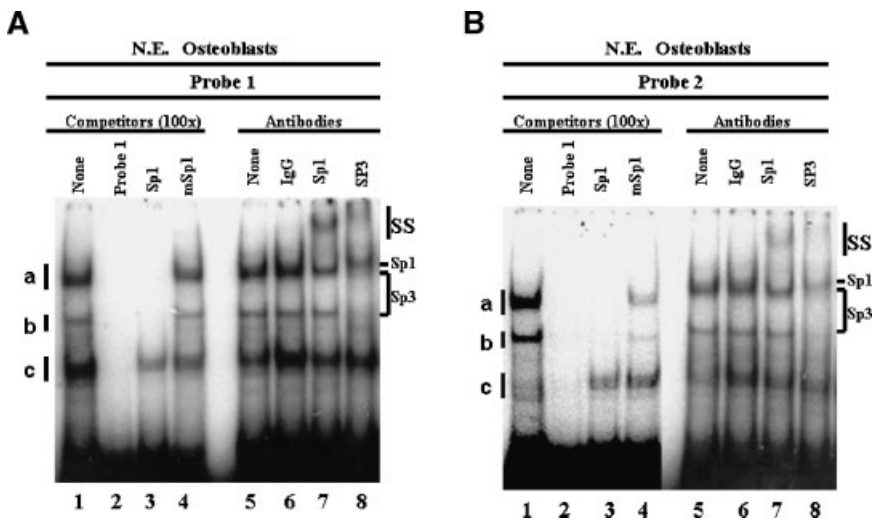

Fig. 5. Identification of osteoblast transcription factors binding to Probes 1 and 2 using competition and supershift assays. **A**: Competition and supershift assays with Probe 1. For competition assays, a 100-fold excess amount of unlabeled Probe 1, oligonucleotide containing Sp1 consensus sequence (Sp1, 5′-ATTCGATCGGGGCGGGGCGAGC-3′) and oligonucleotide containing mutations in Sp1 consensus sequence (mSp1, 5′-ATTCGATCGGTTCGGGGCGAGC-3′ and the mutations are underlined) were used. For supershift assays, 4 μg control IgG or 4 μg Sp1 and Sp3 antibodies were used. **B**: Competition and supershift assays with Probe 2. The assays were performed as in (A). NE, nuclear extracts; SS, supershift.

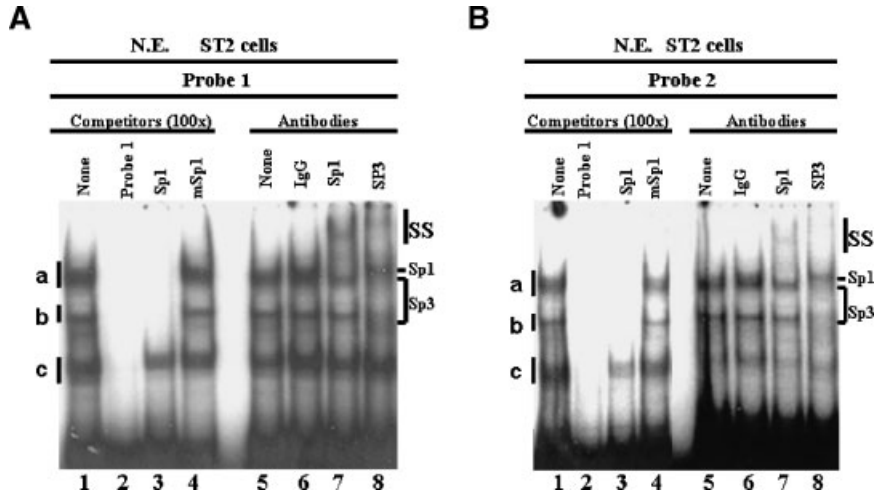

Fig. 6. Identification of stromal cell transcription factors binding to Probes 1 and 2 using competition and supershift assays. **A**: Competition and supershift assays with Probe 1 using nuclear extracts from ST2 cells. The assays were performed as in Figure 5A. **B**: Competition and supershift assays with Probe 2 using nuclear extracts from ST2 cells. The assays were performed as in Figure 5A. NE, nuclear extracts; SS, supershift.

(mProbe 2), in which 4 Gs in the core binding TGGGAGGG were converted to 4 Ts (Fig. 7A). EMSA showed that the binding of Sp1/Sp3 to mProbe 1 was significantly reduced compared to Probe 1 (Lanes 1 and 2, Fig. 7B,C), indicating that the chosen mutations in mProbe 1 are sufficient to eliminate the binding capacity of the sequence. Similarly, our data also demonstrated that the binding of Sp1/Sp3 to mProbe 2 was greatly reduced compared to Probe 2 (Lanes 3 and 4, Fig. 7B,C), revealing that the chosen mutations in mProbe 2 are capable of abolishing the binding capacity of this Sp1/Sp3 site.

We introduced the same mutations individually in our longest reporter construct, RL (−953), resulting in reporter constructs M1 and M2, or in combination, giving rise to a reporter construct M3 (Fig. 8A). We then transiently transfected these mutant constructs as well as RL (−953) as control into osteoblasts (Fig. 8B) or ST2 cells (Fig. 8C). The transfection data indicated that both Sp1/Sp3 binding sites are involved in the RANKL basal transcription in osteoblasts and ST2 cells. However, the Sp1/Sp3 site present in Probe 1, which is located from −151 to −144 (Fig. 7A), plays a significant role in the RANKL basal transcription in both osteoblasts and ST2 cells since the mutation of this site (M1) led to profound reduction in the RANKL basal transcription in osteoblasts as well as ST2 cells (Fig. 8B,C). By comparison, the second Sp1/Sp3 site, which is present in Probe 2 and located from −124 to −117, exerts a moderate effect on the RANKL basal transcription in both cell types since the mutation of this site (M2) gave rise to less reduction in the RANKL basal transcription in both osteoblasts and ST2 cells than that of M1 (Fig. 8B,C). Most significantly, the mutation of both sites (M3) abolished the RANKL basal transcription in the two cell types (Fig. 8B,C), indicating that the two Sp1/Sp3 sites together play an essential role in the RANKL basal transcription in osteoblasts and ST2 cells.

DISCUSSION

In the present study, we sought to investigate the molecular mechanism controlling the RANKL basal transcription in osteoblasts and stromal cells. Using a series of EMSA/competition/supershift assays as well as transient transfection assays, we elucidated that the RANKL basal transcription in osteoblasts and stromal cells is primarily regulated by two sequences in the proximal RANKL promoter region (one from −151 to −144 and the other from −124 to −117, relative to the transcription start site). Moreover, we found that these two sequences regulate the RANKL basal transcription in both osteoblasts and stromal cells by binding the transcription factors: Sp1 and Sp3.

Sp1 and Sp3 belong to the Sp1-like/KLF family of the transcription factors and members of this family are implicated in the regulation of diverse cellular functions such as cell proliferation, differentiation, and apoptosis [Bouwman and Philipsen, 2002; Kaczynski et al., 2003].

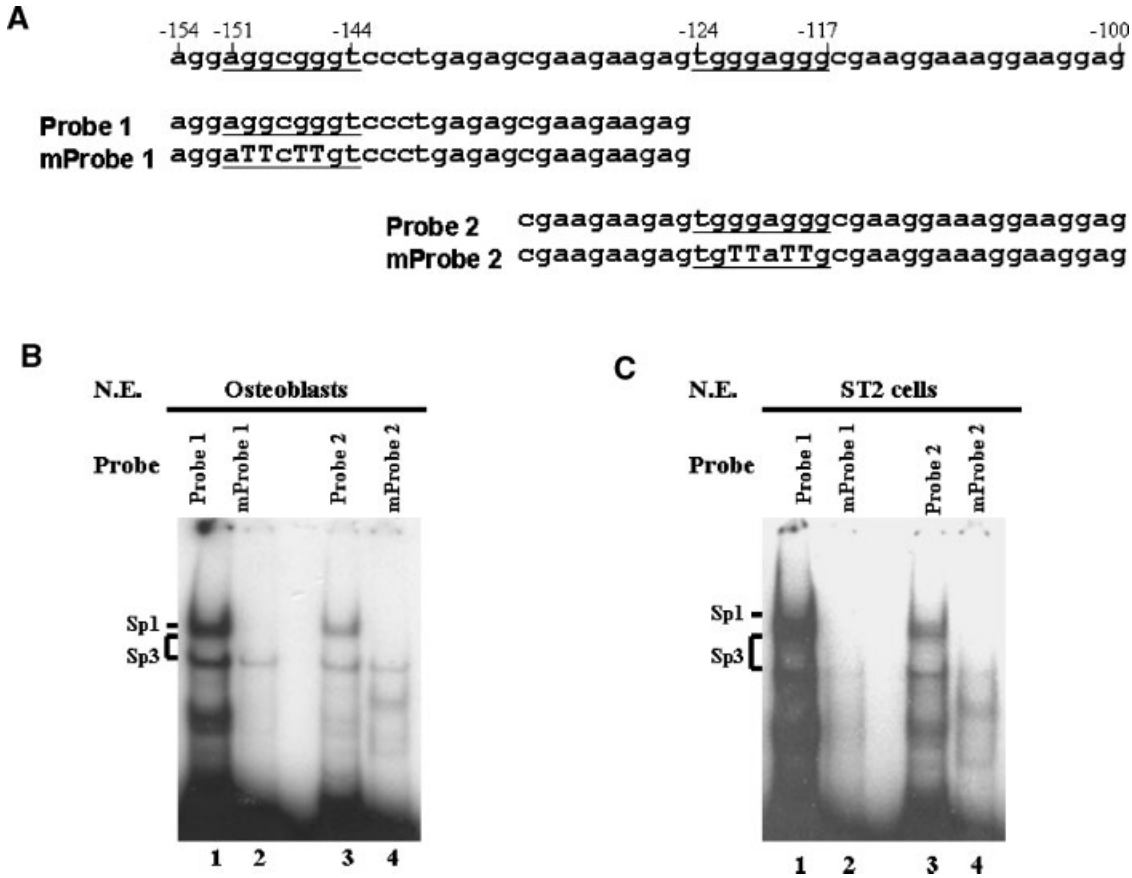

Fig. 7. Identification of the mutations in Probes 1 and 2 that can block the binding of Sp1 and Sp3. **A**: Diagram of the mutant Probes: mProbe 1 and mProbe 2. The mutations introduced are shown in capital letters. **B**: EMSA with Probe 1, mProbe 1, Probe 2, and mProbe 2 using nuclear extracts from osteoblasts. **C:** EMSA with Probe 1, mProbe 1, Probe 2, and mProbe 2 using nuclear extracts from ST2 cells. NE, nuclear extracts.

The carboxyl termini of the Sp1-like/KLF family proteins are highly conserved and contain three Cys2His2 zinc finger motifs that form the DNA-binding domain of these transcription factors. The amino termini of the Sp1-like/KLF family members contain the transcriptional activation domains and are less conserved among the family members [Bouwman and Philipsen, 2002; Kaczynski et al., 2003]. The transcription factors belonging to this family often take part in regulating the transcriptional activation of genes that possess GC-rich promoters [Bouwman and Philipsen, 2002]. Specifically, it has been shown that the Sp1-like/KLF family members recognize the GC-box (GGGGCGGGG) and GT-box (GGTGTGGGG) usually found in the GC-rich promoters [Bouwman and Philipsen, 2002].

Our initial characterization of the RANKL promoter located a 100-bp GC-rich region (62% GC) that mediates the RANKL basal transcription in both osteoblasts and stromal cells (Fig. 3). By performing EMSA with five overlapping oligonucleotides (Probes 1–5, Fig. 4), we found that Probe 1 bound nuclear proteins from both osteoblasts and stromal cells with the highest affinity (Fig. 4). In line with the finding, Probe 1 contains a sequence GaGGCGGGt, which more resembles the GC-box (GGGGCGGGG) than any other sequences in the 100-bp region. In addition, Probe 2 showed less affinity for nuclear proteins than Probe 1, reflecting that the overall structure of the core sequence (tGGGaGGG) in Probe 2 is less similar to the GC-box than the one present in Probe 1. Although three other oligonucleotides (Probes 3–5) also contain numerous GC-rich sequences, they are much less similar to the GC-Box. Consistently, these three oligonucleotides exhibited low affinity for nuclear proteins from

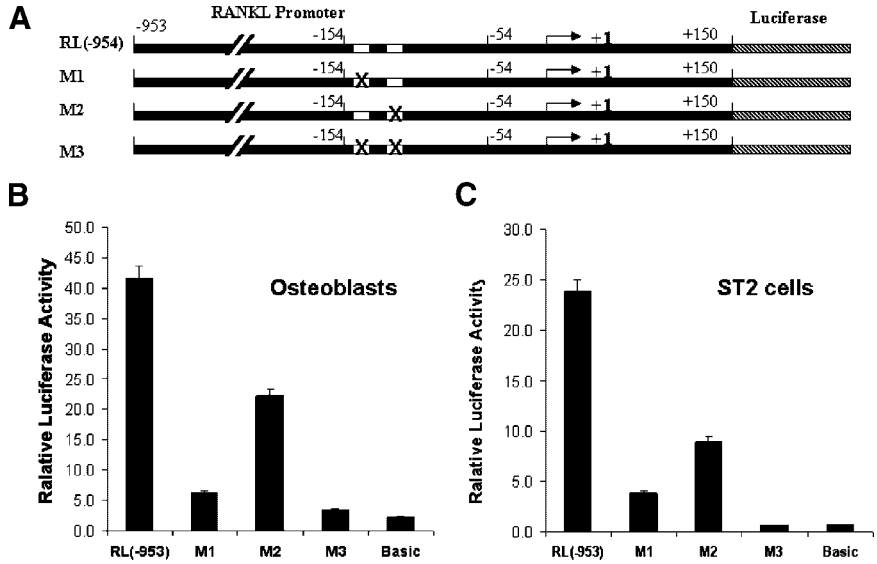

Fig. 8. Mutation of the two Sp1/Sp3 binding sites in the RANKL promoter blocks the RANKL basal transcription. **A**: Schematic diagram of three mutants (M1, M2, and M3). **B**: Transfection assays with RL (−953), M1, M2, and M3 using primary osteoblasts. **C**: Transfection assays with RL (−953), M1, M2, and M3 using ST2 cells. The experiments were repeated three times and one representative result is shown. Each bar is the mean of three replicates ± SD.

osteoblasts and ST2 cells (Fig. 4). Consistent with the very low affinity of the three oligonucleotides for nuclear proteins revealed by EMSA, subsequent functional studies demonstrated that the RANKL basal transcription is regulated primarily by the two sequences found in Probes 1 and 2 (Fig. 8).

Sp1 and Sp3 have been previously shown to play important roles in the differentiation and function of osteoblasts. The expression of integrin β5 subunit, an adhesion molecule mediating osteoblast-bone matrix interaction, is regulated by both Sp1 and Sp3 [Feng et al., 2000; Lai et al., 2000]. In addition, two Sp1 binding sites are implicated in the expression of the receptor for the human PTH/PTH-related protein in osteoblasts [Manen et al., 2000]. The significant role of Sp1 in bone metabolism was further supported by the finding that a COL1A1 Sp1 site polymorphism predisposes to osteoporotic fractures [Mann et al., 2001]. In stromal cells, Sp1 was shown to regulate the estrogen-dependent downregulation of the gene for M-CSF, a critical factor for osteoclastogenesis [Srivastava et al., 1998]. Our findings that Sp1 and Sp3 modulate the basal transcription of *RANKL* gene in both osteoblasts and stromal cells reinforces the functional involvement of the Sp1-like/KLF proteins in bone metabolism.

We took the investigation of the basal transcription of *RANKL* gene in osteoblasts and stromal cells as the first step in our effort to fully elucidate the molecular mechanism by which *RANKL* gene is regulated by various factors in the bone cells. The identification of the essential role for Sp1 and Sp3 in the RANKL basal transcription has raised the question regarding whether Sp1 and Sp3 are implicated in the regulation of RANKL gene expression by the factors such as IL-1, TNF-α, 1α,25(OH)2 vitamin D3, dexamethasone, or TGF-β, especially given the previous findings that Sp1 interacts with numerous transcription factors to regulate gene transcription [Bouwman and Philipsen, 2002]. The most relevant transcription factor is NF-κB. IL-1 and TNF-α are two potent activators of NF-κB [Dunne and O'Neill, 2003; Wajant et al., 2003] and Sp1 has been shown to interact with NF-κB to synergistically activate gene transcription [Perkins et al., 1993, 1994; Majello et al., 1994; Pazin et al., 1996]. Thus, one possibility is that IL-1/TNF-α-activated RANKL gene activation may involve the functional interaction between NF-κB and Sp1. Moreover, Sp1 and Sp3 have been implicated in the steroid hormone-dependent regulation of several genes in the endometrium [Krikun and Lockwood, 2002]. Whether 1α,25(OH)2 vitamin

D3 and dexamethasone-induced activation of *RANKL* gene in osteoblasts and stromal cells involves Sp1 and Sp3 remains to be examined. Finally, it has been shown that Smad3, a TGF-β-activated signaling molecule, is able to interact with Sp1 to modulate gene activation in response to TGF-β [Inagaki et al., 2001], suggesting a potential crosstalk between TGF-β signaling and the action of the Sp1-like/KLF family proteins. Although these proposed mechanisms are speculative, they remain to be reasonable hypotheses to be tested in our future efforts to fully elucidate the mechanism by which these factors regulate RANKL gene expression in osteoblasts and stromal cells.

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