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Transcriptional regulation of human RANK ligand gene expression by E2F1

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Abstract

Receptor activator of nuclear factor kappa B ligand (RANKL) is a critical osteoclastogenic factor involved in the regulation of bone resorption, immune function, the development of mammary gland and cardiovascular system. To understand the transcriptional regulation of RANKL, we amplified and characterized a 1890 bp 5'-flanking sequence of human RANKL gene (-1782 bp to +108 bp relative to the transcription start site). Using a series of deletion mutations of the 1890 bp RANKL promoter, we identified a 72 bp region (-172 to -100 bp) mediating RANKL basal transcriptional activity. Sequence analysis revealed a putative E2F binding site within this 72 bp region in the human RANKL promoter. Over expression of E2F1 increased RANKL promoter activity, while down regulation of E2F1 expression by small interfering RNA decreased RANKL promoter activity. RT-PCR and Enzyme linked immunosorbent assays (ELISA) further demonstrated that E2F1 induced the expression of RANKL. Electrophoretic gel mobility shift assays (EMSA) and antibody competition assays confirmed that E2F1 proteins bind to the consensus E2F binding site in the RANKL promoter. Mutation of the E2F consensus binding site in the RANKL promoter profoundly reduced the basal promoter activity and abolished the transcriptional modulation of RANKL by E2F1. These results suggest that E2F1 plays an important role in regulating RANKL transcription through binding to the E2F consensus binding site.

Keywords

RANK; RANKL; E2F1; Transcriptional regulation

Introduction

Receptor activator of nuclear factor kappa B ligand (RANKL) is a member of the tumor necrosis factor (TNF) family and exists naturally as both a membrane associated and soluble protein by proteolytic cleavage [1;2]. The expression of RANKL is detected in stromal fibroblasts, osteoblasts, T cells and malignant tumor cells [3;4]. RANKL plays critical roles

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in various biological events such as bone remodeling [5;6], immune response [3;7;8], development of the mammary gland [9] and cardiovascular system [10].

Many hormones and cytokines regulate bone homeostasis through modulating RANKL expression. Several osteotropic factors/resorption stimuli including 1, 25-(OH)₂-D₃, parathyroid hormone (PTH), prostaglandin E₂, dexamethasone, interleukin-1 (IL-1) stimulate osteoclasts through upregulation of RANKL expression [2;11]. Recently, the human and mouse RANKL gene 5'-flanking regions have been isolated [12]. Several studies demonstrated that 1, 25-(OH)₂-D₃ induced the expression of RANKL through functional vitamin D response element (VDRE) motifs in both human and mouse RANKL promoter [12;13]. Similarly, basic fibroblast growth factor (b-FGF) stimulated RANKL expression through modulating the binding of the heat shock factors to the heat shock factor responsive elements in human RANKL gene promoter region [14]. Moreover, the study of the basal transcription of murine RANKL gene in osteoblasts and bone marrow stromal cells revealed that transcription factors sp1 and sp3 played a key role in RANKL basal transcription through functional sp1 binding sites [15].

E2F1 belongs to E2F family of transcription factors. Members of this family are central modulators of important cellular events, including cell cycle progression, apoptosis and DNA damage checkpoint control [16;17]. It has been demonstrated that E2F target genes all contain the E2F consensus binding site in their promoters [T/C]TT[C/G][G/C]CG[C/G] [18].

RANKL gene regulation is critical to our understanding of the functions of RANKL involved in different aspects of biological processes. To determine the common regulatory elements involved in the transcriptional regulation of RANKL, we cloned and characterized the 5'-flanking sequences of human RANKL gene. A 72 bp region mediating RANKL transcription activity and the presence of E2F consensus binding site within this 72 bp RANKL promoter region were defined. We further identified E2F1 as a critical transcription factor that regulates RANKL gene transcription.

Experimental procedures

Cell culture

Human SaOS-2 osteosarcoma cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). The cells were grown at 37° C in 5% CO₂ and 95% air.

Chemicals and reagent

The antibody against human E2F1 was purchased from Santa Cruz Biotechnology. The E2F1 overexpression vector was generously provided by Dr. David Goodrich from Roswell Park Cancer Institute with permission from Dr. David Johnson from MD Anderson Cancer Center. ShRNAs to E2F1, E2F2 and E2F3 were purchased from Open Biosystems, Huntsville, AL.

Human RANKL promoter luciferase reporter gene constructs

A 1890 bp human RANKL promoter (from -1798 to +108 bp in relation to the transcription start site designated as +1) was amplified from human BAC clone RP11-86N24 using primers spanning a RANKL core promoter region (Forward, 5'-gtgggttagttgtataacac-3', Reverse, 5'-gtcgtctctgatgtggttcttg-3'). The amplified fragment was subsequently subcloned into a firefly luciferase expression vector pGL2-basic (Promega, Madison, WI). The authenticity of this construct pGL2-RANKL-promoter was confirmed by matching its sequence against human RANKL promoter. Similarly, a series of deletion truncations of pGL2-RANKL core promoter were generated by PCR amplification. The authenticity of these constructs was determined by DNA sequencing.

Transient transfection and luciferase reporter study

SaOS-2 cells were transiently transfected using transfection reagent Tfx-20 (Promega). SaOS-2 cells were seeded in 24 well plates (5×10^4 cells/well) and grown to 70-80% confluence. Each of RANKL promoter-luciferase construct was co-transfected with pRL-TK (TK promoter-Renilla luciferase construct as internal control) into SaOS-2 cells. Briefly, 490 ng of a RANKL construct and 10 ng of pRL-TK were mixed in 200 μ l of serum free RPMI-1640 in a 1.5 ml tube for each well of 24-well plates. After incubation at room temperature for 30 min, the DNA/Tfx mixture was added to each well. The DNA-Tfx complex was replaced by complete medium containing 10% FBS following incubation for 1 h. The luciferase activity was determined 24-48 hrs after transfection using a dual luciferase reporter assay system (Promega). Cell lysates (20 μ l/well) were used for measurement of luciferase activity in a luminometer by first mixing the cell lysates (20 μ l) with 20 μ l of luciferase assay reagent for measuring firefly luciferase activity and subsequently adding 20 μ l of Stop-Glo reagent for measuring Renilla luciferase activity. Data were normalized to Renilla luciferase activity (internal control) as arbitrary units.

Chromatin immunoprecipitation assay

The E2F1 and protein complexes were cross-linked inside the SaOS-2 cells by the addition of formaldehyde (1% final concentration) to the cells in culture. Whole cell extracts were prepared using sonication and an aliquot of the cross-linked protein complexes were immunoprecipitated by incubation with either the E2F1 specific antibody (Santa Cruz Biotechnologies, CA) or IgG antibody overnight at 4 °C with rotation. Chromatin-antibody complexes were isolated from solution by incubation with protein G-Sepharose beads for 1 h at 4°C with rotation. The Sepharose-bound immune complexes were washed and eluted from beads with elution buffer (1% SDS and 0.1 M NaHCO₃). Elute was heated at 65°C for 4hr to reverse the formaldehyde cross-linking and DNA extracted. DNA samples from chromatin immunoprecipitation preparations were analyzed by PCR using primers spanning RANKL gene in the region of promoter (Forward, 5'-CTTGGAGGGCCCCCATGAAGGA, Reverse, 5'-CCCTTCTTGTCTGCGGCCAACT).

Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells with TRIzol reagent (Life Technologies, Rockville, MD). One microgram of total RNA was reverse transcribed with oligo (dT) and Im-Prom II™ reverse transcriptase (Promega, Madison, WI). The resulting cDNA was subjected to PCR analysis using gene specific primers for human RANKL: Forward, 5'-GCTTGAAGCTCAGCCTTTTGCTCAT-3', reverse, 5'-GGGGTTGGAGACCTCGATGCTGATT-3'. Actin: forward, 5'-CCCAGCCATGTACGTTGCTA-3', reverse, 5'-AGGGCATACCCCTCGTAGATG-3'. The PCR amplification was performed by incubating the samples at 95°C for 5 min, followed by 40 cycles of 1 min at 95°C, 30 s at 60°C and 30 s at 72°C, with a final extension for 10 min at 72°C. The amplified products were separated by gel electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

Site-directed mutagenesis

Point mutations were introduced to the 1890 bp pGL2-RANKL promoter construct at the putative E2F binding site (-117 bp) using the QuickChange™ site-directed mutagenesis kit (Stratagene, LaJolla, CA). A 2 nt substitution (AT) was incorporated at the underlined conserved G/C core of this E2F binding site (TTTCGCCC) as this substitution has been shown to eliminate E2F binding [19]. PCR amplification was performed using the following condition: 95°C for 30s, 1 cycle; 95°C for 30s, 55°C for 1 min and 68°C for 7.4 min, 14 cycles and 4°C park. The PCR product was treated with 10 U Dpn I at 37°C for 1 hr and used for

transformation of competent cells as described in the instruction manual. The mutant plasmid was sequenced to confirm the mutations.

Western Blot analysis

The protein extracts were resolved on 10% SDS-PAGE. Proteins were then transferred to nitrocellulose membrane. After blocking overnight at 4°C in 5% milk in PBS- 0.1% Tween 20, membranes were incubated for 1 h at room temperature with anti-E2F1 rabbit polyclonal antibody or anti-pol II (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 1% milk in PBS-Tween-20. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system.

Electrophoretic gel mobility shift assays (EMSA)

The nuclear extracts from cells were prepared and 10 µg of nuclear protein was incubated with binding buffer containing 10 mM Hepes pH 7.9, 400 mM NaCl, 1 mM EDTA and 40% glycerol and 1 µg/reaction poly (dI-dC) with 10⁵ cpm of the γ -p³²-ATP labeled oligonucleotides located in the regions of the RANKL promoter between -117 bp to -110 bp containing either the wild-type (TTTCGCCC) or mutant (TTTCGATC) E2F1 consensus binding site for 30 min at room temperature, respectively. The protein-DNA complexes were resolved on a 5% non-denaturing polyacrylamide gel. The gel was dried, and autoradiographed using Storm 840 Imaging system (Molecular Dynamics, Sunnyvale, CA).

Enzyme linked immunosorbent assays (ELISA)

The secretion of RANKL protein by LNCaP cells was determined by ELISA. Cells were transiently transfected with either E2F1 expression vector or shRNA against E2F1 and the supernatant was collected. RANKL protein was detected by ELISA with the use of anti-RANKL as primary antibody according to the manufacturer's instruction (e-Bioscience, CA). RANKL expression was normalized to the cell number of the samples.

Results and Discussion

Characterization and identification of minimal regulatory regions of RANKL promoter

We amplified a 1890 bp 5'-flanking sequence of RANKL gene (-1782 to +108 bp relative to the transcription start site) from human BAC clone RP11-86N24 and cloned into a firefly luciferase expression vector pGL2-basic. The amplified 1890 bp of the 5'-flanking regions of RANKL gene was sequenced and was identical to the one deposited in Genbank (AF544022). To examine whether the pGL2-RANKL promoter luciferase plasmid is transcriptionally active, we performed transient transfection assays using the 1890 bp pGL2-RANKL promoter construct in SaOS-2 cells. The RANKL promoter exhibited high promoter activity (Fig 1A). To further define the regulatory elements in the RANKL promoter, a series of deletion truncations of pGL2-RANKL promoter were generated by PCR amplification (Fig. 1A). The promoter activity of the various deletions of RANKL promoter was tested in SaOS-2 cells. The RANKL promoter region (-302 to +108 bp) harbored very high promoter activity (Fig. 1A). Further deletion to the -172 site still retains majority of the RANKL promoter activity. Further deletion to the -100 bp site almost completely abolished RANKL promoter activity. Thus, the 72 bp region located between -172 and -100 bp sites contains critical regulatory elements in transcriptional regulation of RANKL (Fig. 1A).

E2F1 regulates RANKL promoter activity

To further identify the transcription factors that regulate RANKL transcription activity, the promoter sequences were subjected to search for transcription factor binding sites. A putative consensus E2F binding site (TTTCGCCC at -118) was identified within the 72 bp (-172 to

-100 bp) RANKL promoter region by computer analysis program (TFSEARCH). This putative binding site for E2F is the only consensus binding site for E2F family in the entire 1890 bp RANKL promoter region (Fig. 1B).

The identification of E2F consensus binding site in RANKL promoter suggests that members of E2F family may regulate RANKL transcriptional expression. To evaluate whether RANKL promoter activity was regulated by E2F, we first tested the effects of overexpression of E2F1 on RANKL promoter activity. Co-expression of E2F1 with the 1890 bp pGL2-RANKL gene promoter luciferase reporter plasmid in SaOS-2 cells increased the RANKL luciferase activity in a dose dependent manner (Fig. 1C). In contrast, co-expression of siRNA against E2F1 with the 1890 bp pGL2-RANKL promoter in SaOS-2 cells decreased the promoter activity to about 50% (Fig. 1D). On the other hand, co-expression of siRNA against E2F2 and E2F3 decreased the promoter activity of RANKL only about 20% or 10% (Fig. 1D). These data suggest that E2F binding site identified in the RANKL promoter region is functional and E2F1 plays an important role in modulating RANKL gene expression.

E2F1 binds to RANKL gene promoter

We further determined whether E2F1 binds to RANKL gene promoter using EMSA and ChIP assays. Two oligonucleotides were synthesized. Wild type oligonucleotide contained the E2F consensus binding site (TTTCGCCC) in the context of RANKL promoter, the mutated oligonucleotide contained a two nucleotide substitution (AT) incorporated at the conserved CG core of the E2F consensus site (TTTCGATC). This two nucleotide substitution has been shown to be able to eliminate the binding of E2F1 to the E2F consensus binding site [19]. These two oligonucleotides were radiolabeled and incubated with the nuclear protein from SaOS2 cells. Nuclear protein bound to the wild type oligonucleotide and formed the protein-DNA complex (lane 2, Fig. 2A). In contrast, there is no protein-DNA complex formation when nuclear extract was incubated with the mutated oligonucleotides (lane 6, Fig. 2A). The composition of this protein-DNA complex was examined by the addition of antibodies against E2F1 or normal IgG. The addition of polyclonal anti-E2F1 antibody (α E2F1) reduced the intensity of the binding of the protein-DNA complex (lane 3 and 4, Fig. 2A). Conversely, the addition of normal IgG did not have any observable effect on the intensity of the protein-DNA complex. These results indicated that E2F1 interacted with RANKL promoter via the E2F consensus binding site. Consistent with this conclusion, the binding of E2F1 to RANKL promoter was further confirmed by the use of chromatin immunoprecipitation assay. Soluble chromatin from SaOS2 cells was prepared and incubated with polyclonal anti-E2F1 antibody or normal IgG. The antibody bound DNA was analyzed by PCR using primers spanning the region (-302 to +108) within the RANKL promoter. As shown in Fig. 2B, anti-E2F1 antibody was able to immunoprecipitate the RANKL promoter region whereas normal IgG failed to pull the region down. Collectively, these data confirmed that E2F1 protein binds to the RANKL promoter.

E2F1 induces the expression of RANKL

To further examine whether E2F1 affects the expression of RANKL, the mRNA levels and protein secretion of RANKL were determined in SaOS2 cells after transient transfection with either E2F1 expression vector or shRNA against E2F1 by RT-PCR and ELISA. Transient expression of E2F1 induced RANKL mRNA expression and protein secretion of RANKL compared to vector control (Fig. 3A, B). In contrast, the use of shRNA against E2F1 down regulated the levels of mRNA and the protein secretion of RANKL (Fig. 3A, B). These results demonstrated that E2F1 transactivates the expression of RANKL.

E2F1 is functionally involved in the RANKL transcription

We determined whether E2F binding site in the RANKL promoter is functionally involved in regulating RANKL basal transcription through introducing mutations into the E2F1 consensus binding sequence which has been shown to eliminate the binding of E2F transcription factors to this sequence [19]. Two nucleotide mutation (AT) was incorporated to the 1890 bp pGL2-RANKL promoter luciferase plasmid construct E2F consensus sequence (TTTCGCC) at the C/G core through site-directed mutagenesis and the sequence was confirmed by DNA sequencing. We then transiently transfected the mutant promoter construct as well as the wild type 1890 bp pGL2-RANKL promoter gene construct into SaOS-2 cells. As demonstrated before, wild type 1890 bp pGL2-RANKL promoter showed very high promoter activity. However, the E2F binding site mutated promoter lost almost 50% of the transcription activity of RANKL promoter (Fig. 4A). These data suggest that E2F consensus binding site plays a significant role in RANKL transcription. Furthermore, we tested the functionality of E2F binding site through co-expression of E2F1 along with either wild type or mutated 1890 bp pGL2-RANKL promoter. As demonstrated in Fig. 1C, E2F1 stimulated wild type RANKL promoter activity. In contrast, E2F1 had no appreciable effect on the mutated RANKL promoter activity (Fig. 4B). These results indicated that the mutation in the E2F consensus binding site blocked the binding of E2F to the RANKL promoter, thus abolishing the transactivating effect of E2F1.

In summary, we have cloned a 1890 bp 5'-flanking region of human RANKL gene and identified a 72 bp region (-172 to -100 bp) in the RANKL promoter that regulates RANKL transcriptional activity. Further analysis showed that E2F1 regulates RANKL transcriptional expression.

Acknowledgments

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Fig. 1. Transcriptional regulation of RANKL gene promoter

A. Mapping of the domain of RANKL promoter mediating transcription activity using transient transfection assay in SaOS-2 cells. Various constructs were generated by PCR amplification. The construct length (left) and its relative luciferase activity (right) are indicated. Luciferase activity was measured using a dual luciferase reporter system as described under “Materials and Methods”. Data were derived from a representative experiment in triplicate after normalization to *Renilla* luciferase activity (internal controls). Each bar is the mean $\pm$ S.D.

B. Sequences of the 72 bp (-172 to -100 bp) of RANKL regulatory region. E2F consensus binding site was indicated in bold.

C. E2F1 regulated RANKL promoter activity. pGL2-RANKL1890 promoter luciferase construct was transfected into SaOS-2 cells. The promoter activity was examined in the presence and absence of E2F1 expression vector (E2F1 CMV).

D. pGL2-RANKL1890 promoter luciferase construct was transfected into SaOS-2 cells. The promoter activity was examined in the presence and absence of the shRNA against E2F1, E2F2, and E2F3, respectively. Luciferase activity was measured using a dual luciferase reporter system as described under “Materials and Methods”. Data were derived from a representative experiment in triplicate after normalization to *Renilla* luciferase activity (internal controls). Each bar is the mean $\pm$ S.D.

Fig. 2.

A. E2F1 specifically binds to RANKL promoter. The binding of E2F1 to RANKL promoter was analyzed by the use of wild type E2F1 probe (E2F1 probe-WT) or mutant probe (E2F1 probe-M) following the addition of anti-E2F1 (α E2F1) and normal IgG antibodies. Lane 1, free probe; lanes 2 to 5, nuclear extracts (10 μ g) were incubated with wide type E2F1 probe; lane 3 to 5, antibodies (1, 2 μ g α E2F1 and 2 μ g IgG) were added to the incubation of nuclear extract with E2F1 probe-WT; lane 6, nuclear extracts (10 μ g) were incubated with mutant E2F1 probe; lane 7 to 9, antibodies (2 μ g α E2F1 and 2 μ g IgG) were added to the incubation of nuclear extract with E2F1 probe-M). **B.** E2F1 interacted with RANKL promoter. Chromatin immunoprecipitation assays were performed by the use of either polyclonal anti-E2F1 antibody

or normal IgG. PCR amplification products were examined for either anti-E2F1 antibody or normal IgG enriched DNA template. Input was shown as a control.

Fig. 3. E2F1 regulated the expression of RANKL

A. E2F1 expression vector (E2F1 CMV) or siRNA against E2F1 (E2F1 siRNA) was transiently transfected into Saos2 cells. Total RNA was extracted and the mRNA level of RANKL was determined by RT-PCR. Actin was used as a RNA loading control. **B.** E2F1 expression vector (E2F1 CMV) or siRNA against E2F1 (E2F1 siRNA) was transiently transfected into Saos2 cells. The media was collected and the secretion of RANKL in the media was determined by ELISA with the use of anti-RANKL antibody as primary antibody. **C.** E2F1 protein expression. E2F1 expression vector (E2F1 CMV) or siRNA against E2F1 (E2F1 siRNA) was transiently transfected into Saos2 cells. Nuclear protein was isolated and E2F1 protein expression was determined by Western blot analysis using antibody against E2F1. The expression of Pol II was used as marker for the integrity of the nuclear fraction.

Fig. 4. Mutation at the E2F consensus binding site in RANKL promoter construct abolished the transcriptional regulation by E2F1

A. Mutated RANKL promoter luciferase construct pGL2-RANKL-M was generated through the introduction of a 2 nt substitution at the C/G core of E2F consensus binding site using site-directed mutagenesis. The activities of wild type and mutated RANKL promoter were determined by transient transfection assays in Saos-2 cells. **B.** E2F1 expression vector (E2F1 CMV) was co-expressed along with either wild type or mutated pGL2-RANKL promoter luciferase construct to determine the effect of E2F1 on the expression of RANKL. Luciferase activity was measured using a dual luciferase reporter system as described under “Materials and Methods”. Data were derived from a representative experiment in triplicate after normalization to *Renilla* luciferase activity (internal controls). Each bar is the mean $\pm$ S.D.