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CCAAT/Enhancer Binding Protein Beta Is Up-Regulated in Giant Cell Tumor of Bone and Regulates *RANKL* Expression

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ABSTRACT

Giant cell tumor (GCT) of bone is an aggressive non-cancerous tumor, which consists of multi-nucleated osteoclast-like giant cells, stromal cells, and monocytes. It is believed that stromal cells are the neoplastic component of this tumor. Expression of the receptor activator of nuclear factor kappa B ligand (RANKL) in the stromal cells stimulates the monocytes to form giant multi-nucleated osteoclast-like cells, causing bone over-resorption at the tumor site. Previously, our group has reported the up-regulation of *RANKL* in GCT of bone stromal cells, but the mechanism is unknown. Using stromal cell culture of GCT obtained from patients, we demonstrated the up-regulation of the transcriptional activator CCAAT/enhancer binding protein beta (C/EBPβ). *RANKL* promoter studies revealed that C/EBPβ over-expression induced *RANKL* promoter activity in a dose-dependent manner and a CCAAT-box within the region nt −357/−1 contributed to the basal transcription activity, with a possible C/EBPβ binding element in the region nt −460/−358 leading to further induction. Furthermore, we also showed that C/EBPβ bound to the *RANKL* promoter in GCT stromal cells in vivo by chromatin immunoprecipitation. To conclude, our study has shown that C/EBPβ is a *RANKL* promoter activator in stromal cells of GCT of bone and we have proposed a model in which C/EBPβ plays an important role in the osteolytic characteristics and pathological causes of GCT of bone. J. Cell. Biochem. 110: 438–446, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: STROMAL CELLS; GIANT CELL TUMOR OF BONE; RANKL; C/EBPβ; PROMOTER ANALYSIS

Giant cell tumor (GCT) of bone is an aggressive non-cancerous tumor which seldom metastasizes, but frequently results in local recurrence. This is the most common non-malignant, primary bone tumor reported in Hong Kong, and it usually affects young adults between ages of 20 and 40 [Yip et al., 1996]. The tumor comprises three distinct cell types: (a) hematopoietic mono-nucleated cells, (b) multi-nucleated giant cells, and (c) mesenchyme-derived stromal cells [Wulling and Kaiser, 2003]. Proliferating GCT stromal cells are believed to be the neoplastic component of GCT [Zheng et al., 2001]; these cells express receptor activator of nuclear factor kappa B ligand (RANKL) that stimulates the formation of osteoclast-like multi-nucleated giant cells from monocytes thus leading to over-resorption of bone at the tumor site.

RANKL belongs to the tumor necrosis factor (TNF) family and its expression is normally found in bone marrow stromal or preosteoblast cells, T cells, and dendritic cells [Anderson et al., 1997; Suda et al., 1999]. As part of physiological bone remodeling, RANKL regulates osteogenesis and osteoclastogenesis cooperating with receptor activator of nuclear factor kappa B (RANK) and osteoprotegerin (OPG) [Hofbauer and Heufelder, 2001; Boyce and Xing, 2008]. RANKL binds to RANK expressed on the surface of osteoclast precursor cells and drives them to form active multi-nucleated osteoclasts for bone resorption during bone remodeling. OPG is a natural decoy of RANKL that prevents over-activation and provides negative control. In addition, RANKL activity has been implicated in various physiological processes, such as mammary

Patrick Kwok-Shing Ng and Stephen Kwok-Wing Tsui contributed equally to this study.

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gland development [Fata et al., 2000] and the immune response [Kong et al., 1999], and may contribute to pathophysiological processes in osteosarcoma [Mori et al., 2007a,b], Paget's sarcoma [Sun et al., 2006], breast cancer [Van Poznak et al., 2006], prostate cancer [Chen et al., 2006] as well as vascular disease [Lorenz et al., 2004].

The expression of RANKL in osteoblasts or bone marrow stromal cells is regulated by a number of transcription factors. Sp1 and Sp3 play a role in basal *RANKL* expression [Liu et al., 2005]. Vitamin D_3 induces *RANKL* expression through binding to the Vitamin D-responsive element on its promoter [Kitazawa et al., 2003]. Similarly, cAMP response element-binding protein (CREB), heat shock factor 2 (HSF2), and E2F transcription factor 1 (E2F1) have been shown to bind to their consensus site on the *RANKL* promoter and activate its expression [Roccisana et al., 2004; Bai et al., 2005; Hu et al., 2008]. Previous studies have shown that *RANKL* is over-expressed in the stromal cells of GCT of bone and is a major factor contributing to the osteolytic features of GCT though the formation of multi-nucleated osteoclast-like giant cells [Huang et al., 2000]. However, the mechanism of *RANKL* up-regulation in GCT stromal cells remains unclear.

Here, we report the deregulation of *RANKL* in GCT of bone is related to the up-regulation of transcriptional activator CCAAT/enhancer binding protein beta (C/EBPβ). By truncation and site-directed mutagenesis studies, we show that the CCAAT-box in the *RANKL* promoter is possibly the responsive element of C/EBPβ and contributes to promoter activation.

MATERIALS AND METHODS

CELL CULTURE

All media and reagents used in cell culturing were purchased from Invitrogen. The study was approved by the Ethics Committee of the Chinese University of Hong Kong. Normal osteoblasts and GCT specimens were collected from patients after surgery in the Prince of Wales Hospital, the teaching hospital of the university. As described previously [Cheng et al., 2004], freshly obtained tissues were minced with scissors in low glucose DMEM containing 10% FBS and 100 U/ml penicillin and 100 mg/ml streptomycin. The resultant cell suspension together with small pieces of tissue was transferred to 25 cm^2 flasks for subsequent culturing at 37°C in a humidified, 5% CO_2 incubator. Culture medium was changed every 2–3 days and on reaching confluence, cells were sub-cultured. The stromal cells were used for experiments once no monocytes and giant cells were observed in the culture. Saos-2 (human osteoblast-like cell line) was cultured in the same medium and conditions as GCT stromal cells.

RNA ISOLATION, cDNA SYNTHESIS, AND REAL-TIME PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen) and purified by RNeasy kit (Qiagen). One microgram RNA was used for each reverse transcription reaction using QuantiTect Rev Transcription Kit (Qiagen). Real-time PCR was performed using 2x Power SYBR Green PCR Master Mix (Applied Biosystems) with ABI 7500 Fast Real-time PCR system (Applied Biosystems). The house-keeping gene *GAPDH* was measured for normalization. The primer-pairs of each target gene are listed in Table I. The final primer concentration for *GAPDH* was 125 nM, while that for other genes was 250 nM.

PLASMID

The promoter region (1.8 kb) of the human *RANKL* gene was PCR-amplified from Saos-2 genomic DNA using primers (forward: 5′-GCGAGCTCAAATCATTGTGGGTTTAGTTGTTATAAC-3′; reverse: 5′-GCAAGCTTCCCGGCAGCCCCAACTCT-3′) and cloned into the pGL3-basic vector (Promega) between *Sac*I and *Hin*dIII sites to generate a full-length wild-type *RANKL* promoter luciferase reporter construct. Truncated and site-directed mutated derivatives of *RANKL* promoter were generated by a PCR-based approach and sub-cloned into the same restriction enzyme sites of the pGL3-basic vector (Promega). For the site-directed study, the Cb1 site was modified from 5′-AACTTTCTAAATCC-3′ to 5′-AACgactagtATCC-3′; the Cb2 site was modified from 5′-GGATTGTGAAATTT-3′ to 5′-GGAgactagtATTT-3′; the Cx site was modified from 5′-ATTGGC-3′ to 5′-AggaGC-3′ (mutated nucleotides are in lowercase). The expression vector of C/EBPβ (pCMV6-C/EBPβ) was purchased from OriGene, while the empty expression vector pCMV6 was generated by excising out the C/EBPβ cDNA sequence from the pCMV6-C/EBPβ plasmid at two *Not*I sites, followed by religation, transformation, and sequencing verification.

TRANSIENT TRANSFECTION

One day in advance of transfection, 3×10^4 Saos-2 cells/well were seeded into 24-well plates. On the day of transfection, different combinations of DNA plasmids (total 0.2 μg/well) were transfected with Lipofectamine reagent (0.8 μl/well; Invitrogen) with PLUS reagent (1.2 μl/well; Invitrogen) following the manufacturer's instructions. For the luciferase reporter assay, pRL-CMV *Renilla* luciferase control vector was co-transfected into cells for luminescent signal normalization.

LUCIFERASE REPORTER ASSAY

Transfected cells were harvested 48 h post-transfection. Cells were lysed using Glo-lysis buffer (Promega) and 75 μl lysate was

TABLE I. Primer Sequences for Real-Time PCR

Target gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
CREB	AGCCACTCAGCCGGGTACTA	TAGTGGGTGCTGTGCGAATC
C/EBPβ	ACCAACCGCACATGCAGAT	GCAGAGGGAGAAGCAGAGAGTTT
HSF2	CCAGTATACCGCCTTTCCACTT	AGAGGACAAAACTTCTGATGATGCT
NF-E2	GTCCCAGAAGGAGCTGAGTTCTC	TCCCTCAAGGCAAGCCTCTT
RUNX2	CACTGGCGCTGCAACAAG	CATCGTTACCCGCCATGAC
GAPDH	CGCCCCACTTGATTTTGGA	TTGCCATCAATGACCCCTTCA

transferred to each well of a 96-well plate. The firefly and *Renilla* luciferase activities were measured using the Dual-Glo luciferase assay system (Promega) following the manufacturer's instructions. The data are presented as relative luciferase activity, the ratio of the firefly luminescent value/the *Renilla* luminescent value of the same sample.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

Chromatin immunoprecipitation assay was performed using ChampionChIP One-Day Kit (SABiosciences) according to manufacturer's instruction with few modifications as described below. Briefly, GCT cells were seeded in 100-mm dish and harvested until 80–90% confluence. Chromatins were prepared and fragmented by sonication. Fragmented chromatin was pre-cleared with Protein A beads and normal rabbit IgG (Santa Cruz) at 4°C for 2 h. Ten microliters pre-cleared lysate was saved up as input fraction. Two immunoprecipitation reactions were set up with 1 ml pre-cleared lysate and 4 μg normal rabbit IgG or anti-C/EBPβ antibody (sc-150, Santa Cruz). Reactions were incubated at 4°C for 3 h and followed by adding 60 μl Protein A beads and 4°C incubation for further 1 h. After washing steps, reverse cross-linking and purification of pull-down chromatins, as well as input fraction, were performed as described in manufacturer's manual. Relative amount of C/EBPβ-bound *RANKL* promoter fragment was measured by real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) with forward primer: 5′-GAGGGCGAAAGGAAGGAAG-3′ and reversed primer: 5′-CCCAACTCTTATAAACCGCTTGGA-3′ which flank the CCAAT-box in *RANKL* promoter. Data were analyzed according to manufacturer's suggestion.

STATISTICAL ANALYSIS

Student's *t*-tests were used for the determination of statistically significant differences between samples or treatments. A *P* value less than 0.05 was considered as statistically significant.

RESULTS

PREDICTION OF PUTATIVE HUMAN RANKL-REGULATING TRANSCRIPTION FACTORS

In an attempt to better understand the mechanism of *RANKL* over-expression in GCT stromal cells, we hypothesized that deregulation of RANKL-regulating transcription factor(s) could contribute to the elevated *RANKL* expression in stromal cells of GCT of bone. To test this hypothesis, we performed an in silico analysis of human *RANKL* promoter (1.8-kb upstream from the transcription start site). Among many putative transcription factors predicted, we focused on five transcription factors which have been reported to be involved in bone metabolism: CREB, C/EBPβ, HSF2, nuclear factor erythroid-derived 2 (NF-E2) and runt-related transcription factor 2 (RUNX2). The positions of their predicted binding sites on the *RANKL* promoter are shown in Figure 1.

EXPRESSION LEVELS OF PUTATIVE *RANKL*-REGULATING TRANSCRIPTION FACTORS IN GCT

Expression levels of five selected transcription factors in five stromal cell cultures were examined (Fig. 2A) by real-time PCR. Among the five transcription factors, *C/EBPβ* was significantly up-regulated, while *RUNX2* was marginally up-regulated, in GCT stromal cells compared to the expression in the normal osteoblasts. Not more than twofold change of expression of other three transcription factors, CREB, HSF2, and NF-E2, was observed. To confirm this up-regulation of *C/EBPβ* expression, expression of C/EBPβ compared to normal osteoblasts was examined in more GCT of bone samples (from a total of nine GCT patients). As shown in Figure 2B, the up-regulation ($\geq$2-folds) of *C/EBPβ* expression was observed in 66.7% (six out of nine) of examined stromal cell cultures.

C/EBPβ INDUCES *RANKL* PROMOTER ACTIVITY

In order to test the effect of C/EBPβ on *RANKL* transcriptional activity, a 1.8-kb promoter region of human *RANKL* gene was cloned upstream of a luciferase reporter vector for the luciferase reporter assay in Saos-2 cells. The results showed that *RANKL* promoter activity was significantly induced by 4.7- to 17.4-fold when increasing amounts of C/EBPβ expression vector were co-transfected into the cells (Fig. 3). This result demonstrated that C/EBPβ is able to activate *RANKL* transcriptional activity in a dose-dependent manner.

LOCALIZATION OF THE C/EBPβ-RESPONSIVE ELEMENT ON *RANKL* PROMOTER

There are four C/EBPβ binding sites predicted on the human *RANKL* promoter (Fig. 1). Three of those are C/EBPβ-specific binding sites (at nucleotide (nt) position −1173/−1160, −498/−485, and −391/−378), while the last one is a CCAAT-box (at nt position −56/−51) which can be bound by C/EBP family members [Osada et al., 1996]. In order to find out which is/are functional C/EBPβ-responsive site(s), luciferase reporter vectors with sequential truncated variants of the *RANKL* promoter (Fig. 4A) were generated by a PCR-based approach and their responses to C/EBPβ over-expression were examined in Saos-2 cells. Deletions of the region (−1781/−461) containing distal (Cb3; at nt position −1173/−1160) and middle (Cb2; at nt position −498/−485) C/EBPβ binding sites did not reduce the C/EBPβ-induced *RANKL* promoter activity, while further deletion of the region (−460/−358) containing proximal C/EBPβ binding site (Cb1; at nt position −391/−378) caused significant reduction of C/EBPβ's effect (Fig. 4B). Results suggested that the promoter region nt −357/−1 was sufficient for C/EBPβ-induction of *RANKL* transcription. Interestingly, when the region nt −1781/−579 was deleted, the induction by C/EBPβ doubled, suggesting that possible inhibitory element(s) for C/EBPβ induction may be present within this region (Fig. 4B).

SITE-DIRECTED MUTAGENESIS OF C/EBPβ-RESPONSIVE ELEMENTS ON THE *RANKL* PROMOTER

Since maximum C/EBPβ-induction of *RANKL* promoter activity was observed with the promoter region nt −460/−1 (Fig. 4B), this region was further investigated. Site-directed mutagenesis analysis of putative C/EBPβ binding sites (i.e., Cb2, Cb1, and Cx) was performed. A series of *RANKL*-luciferase reporter vectors containing nt −578/−1 region with single or a combination of mutated C/EBPβ binding sites (Fig. 5A) were assayed in Saos-2 cells. In the validation of basal expression of all mutated constructs (Fig. 5B), we found that

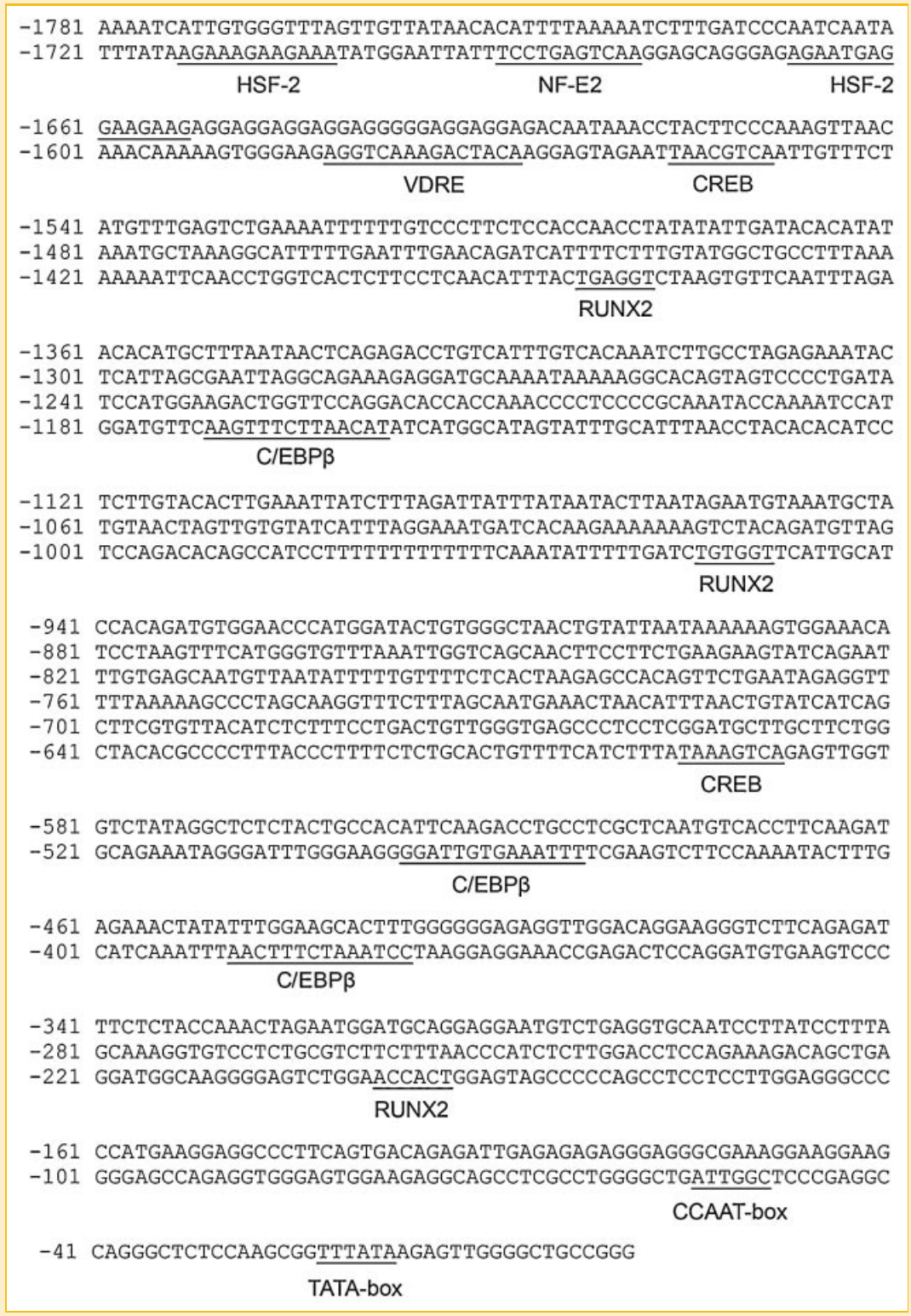

Fig. 1. 1.8-kb 5′-flanking region of human RANKL gene. Putative transcription factor binding sites were identified using web-based software and by literature review. Putative binding sites are underlined and corresponding transcription factors are listed under the sites. HSF2, Heat shock factor 2; VDRE, Vitamin D-responsive element; CREB, cAMP-responsive element-binding protein; RUNX2, Runt-related transcription factor 2; C/EBPβ, CCAAT/enhancer-binding protein β; NF-E2, nuclear factor (erythroid-derived 2).

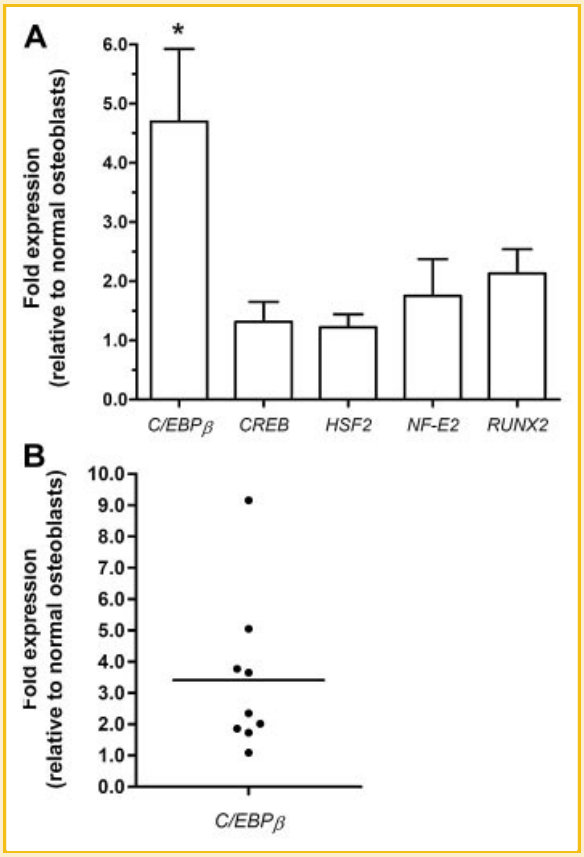

Fig. 2. Expression profile of bone-related transcription factors. A: Expression levels of five transcription factors in five cultures of GCT of bone stromal cells comparing to normal osteoblasts. Data are presented as mean ± SEM. An asterisk indicates significant difference between GCT stromal and normal osteoblasts ($P < 0.01$). B: Expression levels of *C/EBPβ* in 9 GCT stromal cell cultures comparing to normal osteoblasts. The line indicates the mean expression level of the samples compared to normal osteoblasts ($P < 0.05$).

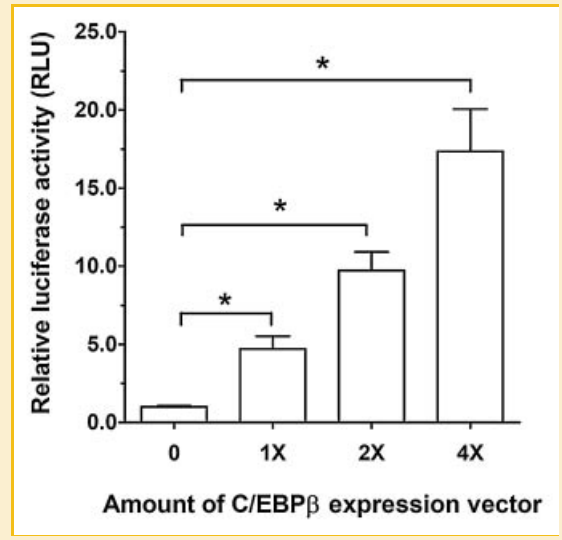

Fig. 3. Effect of C/EBPβ over-expression on *RANKL* promoter activity. Osteoblast-like cells (Saos-2) were transfected with 1.8-kb *RANKL* luciferase reporter vector and empty expression vector or increasing amounts of C/EBPβ expression vector, as well as with *Renilla* luciferase normalization vector. Forty-eight hours post-transfection, cell lysates were harvested and luciferase and *Renilla* luciferase activities were measured. The data are presented as mean ± SD of relative luciferase activity from three independent experiments. Asterisks indicate significant difference between groups ($P < 0.01$).

all of them, no matter single-site or double-site mutation, had compatible activity to the wild-type construct, except for the construct containing the mutated CCAAT-box, which resulted in complete abolishment of the transcriptional activity of the construct in the absence of C/EBPβ. In order to test the importance of these putative C/EBPβ binding sites specifically, they were examined in the presence or absence of C/EBPβ. The result showed only Cx site mutation caused significant reduction of C/EBPβ-induced *RANKL* promoter activity (Fig. 5C).

RECRUITMENT OF C/EBPβ TO *RANKL* PROMOTER IN GCT STROMAL CELLS

To further examine whether the C/EBPβ is recruited into the *RANKL* promoter in GCT stromal cells in vivo, chromatin immunoprecipitation followed by quantitative real-time PCR (ChIP-qPCR) was performed in GCT cells. Primers used in qPCR were flanking the CCAAT-box within the *RANKL* promoter (Fig. 6). Two GCT stromal cell cultures with up-regulated expression of *RANKL* (7.49-fold in GCT culture #1 and 11.32-fold in GCT culture #2, compared to normal osteoblasts) were used. By qPCR, we showed that C/EBPβ bound to the *RANKL* promoter in both examined cultures with different extents. C/EBPβ bound to the *RANKL* promoter in GCT culture #2 is about fivefold more than that in GCT culture #1.

DISCUSSION

Excess resorption of bone minerals is a characteristic of GCT of bone that is believed to be due to increased osteoclastogenesis and osteoclast activation through over-expression of *RANKL* in neoplastic stromal cells [Huang et al., 2000]. However, the mechanism of this elevated *RANKL* expression in the stromal cells is still unknown. Therefore, in the present study, we attempted to find the cause of elevated *RANKL* expression in GCT of bone and thus possibly shed light on the pathology of this tumor. We found that *C/EBP*β was up-regulated in GCT stromal cells collected from patients. *RANKL* promoter activity is highly dependent on the CCAAT-box (at nt −56/−51); and C/EBPβ, as a CCAAT-box binding protein, is able to activate the transcription of the *RANKL* promoter. Moreover, in vivo binding of C/EBPβ to the *RANKL* promoter was observed in GCT stromal cells. Taken together, our findings suggest that elevated expression of C/EBPβ is one of the causes responsible for the *RANKL* up-regulation, thus maintaining the osteolytic feature of GCT of bone.

HSF2 and *CREB* have been demonstrated as transcriptional activators of *RANKL* genes in mammalian stromal/osteoblast cells [Roccisana et al., 2004; Bai et al., 2005] but our results showed that their expression was not changed in GCT stromal cells (Fig. 2A). Thus, our results suggest that up-regulation of *RANKL* in GCT stromal cells is not likely due to the deregulation of these two known

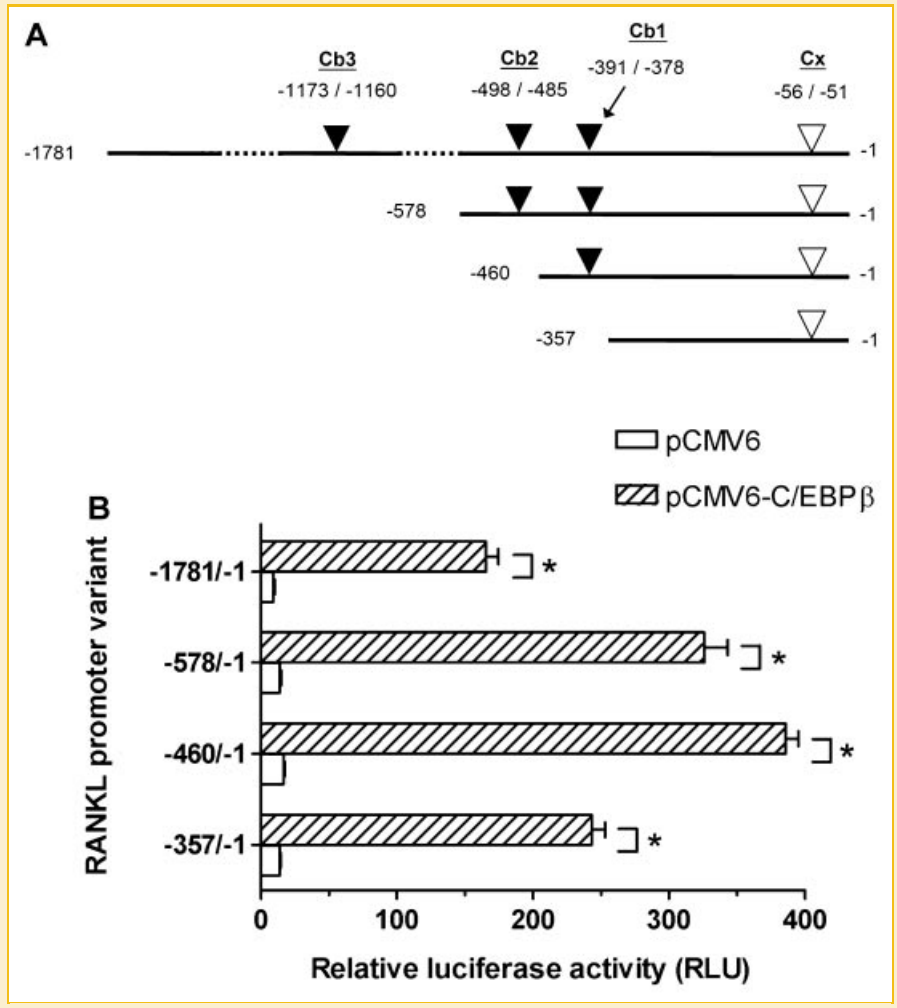

Fig. 4. Deletion analysis of the *RANKL* promoter. A: Schematic diagram of different truncated variants of the *RANKL* promoter. C/EBPβ-specific sites and CCAAT-box are indicated by arrow heads and open arrow heads, respectively. The C/EBPβ-specific sites from nearest to farthest from TSS are designated as Cb1, Cb2, and Cb3, respectively, while the CCAAT-box is designated as Cx. B: Luciferase reporter assay of truncated *RANKL* promoter constructs with empty expression vector (pCMV6) or C/EBPβ expression vector (pCMV6-C/EBPβ). The data are presented as mean ± SD of relative luciferase activity from two independent experiments. Asterisks indicate significant difference between groups ($P < 0.01$).

regulators. The GCT stromal cells are believed to be in osteoblastic lineage, but with an undifferentiated morphology and physiology. Interestingly, we showed that the expression of *RUNX2*, a hallmark osteoblast differentiation marker, was marginally up-regulated (~2-fold compared to normal osteoblasts) in GCT stromal cells. The biological significance of the up-regulation of *RUNX2* in GCT stromal cells is another interesting observation for further investigation. Furthermore, in the latter part of this study, E2F1 has been demonstrated to bind to *RANKL* promoter and induce its expression [Hu et al., 2008]. However, no study of E2F1 expression and its role in GCT stromal cells was found so far, therefore, whether E2F1 involves in the RANKL over-expression in GCT stromal cells might be our next investigation topic.

Although several C/EBPβ-specific binding sites were predicted in the in silico analysis of *RANKL* promoter, the truncation study showed that deletion of all of those specific sites (represented by the clone containing region of nt −357/−1) did not cause drastic reduction of C/EBPβ-mediated induction (Fig. 4B). These results indicate that the major responsive element sufficient for C/EBPβ-mediated induction is located within the region of nt −357/−1. One of the most likely sites is the CCAAT-box located at nt position −56/−51; therefore, site-directed mutagenesis analysis was carried out to test the involvement of this CCAAT-box in *RANKL* promoter regulation. Notably, the mutation of this site totally abolished the transcription activity of the *RANKL* promoter (Fig. 5B) suggesting that the CCAAT-box at nt position −56/−51 is vital for human *RANKL* gene transcription. On the other hand, in vivo binding of C/EBPβ to the *RANKL* promoter in GCT stromal cells was showed and more C/EBPβ binding to *RANKL* promoter was observed in the GCT culture with higher *RANKL* expression (Fig. 6). Based on the fact that C/EBPβ is a CCAAT-box binding protein [Ramji and Foka, 2002], it is conceivable that C/EBPβ is one of the key mediators of *RANKL* over-expression in GCT stromal cells via the binding to the CCAAT-box in the *RANKL* promoter.

In addition, two interesting findings were obtained in the truncation and site-directed mutagenesis analyses. (1) Deletion of

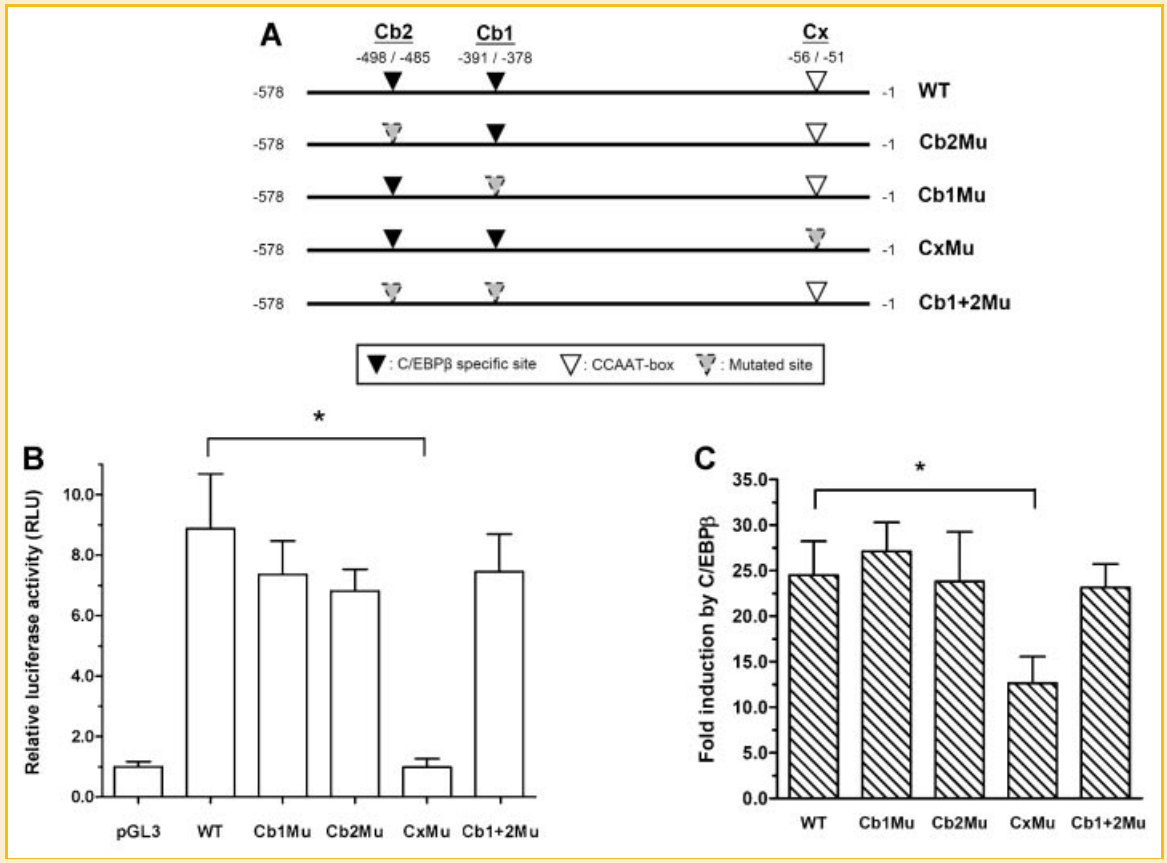

Fig. 5. Site-directed mutagenesis of *RANKL* promoter. A: Schematic diagram of different site-directed mutated *RANKL* promoters. WT is wild-type *RANKL* promoter clone without mutated site; Cb2Mu, Cb1Mu, and CxMu are clones with individually mutated Cb2, Cb1, and Cx site, respectively; Cb1 + Cb2Mu is clone with both mutated Cb2 and Cb1 sites. B: Luciferase reporter assay of site-directed mutated constructs. C: Fold induction of mutated *RANKL* constructs by C/EBPβ. The data were presented as mean ± SD of relative luciferase activity or fold induction from three independent experiments. Asterisks indicate significant difference between pairs ($P < 0.01$).

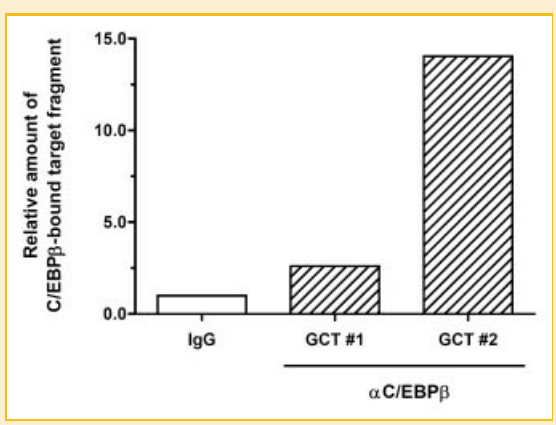

Fig. 6. C/EBPβ bound to *RANKL* promoter in GCT stromal cells in vivo. Chromatins of two GCT stromal cell cultures were prepared and precipitated using C/EBPβ specific antibody (sc-150, Santa Cruz). Relative amount of C/EBPβ-bound *RANKL* promoter fragment were measured using qPCR with primers flanking region nt −10/−120 which contains the CCAAT-box (nt position −56/−51).

the region nt −1781/−579 of the *RANKL* promoter caused a significant increase in C/EBPβ-induced activity, suggesting that inhibitory elements which suppress C/EBPβ-mediated induction may be present within this deletion region. However, the exact suppressing mechanism of C/EBPβ action requires further investigation. (2) Although deletion of the region −460/−358 did not cause abolishment of C/EBPβ's effect (Fig. 4B), it led to significant reduction of *RANKL* promoter activity in the presence of C/EBPβ, suggesting the possible occurrence of a C/EBPβ-responsive element with less inductive capability in this region. As one of the predicted C/EBPβ binding sites within this region, the Cb1 site was examined by site-directed mutagenesis. However, mutation of this site did not lead to significant promoter activity reduction (Fig. 5C). This confirmed that the predicted C/EBPβ at nt position −391/−378 is not a functional binding site. Nevertheless, the possibility of the presence of a non-consensus C/EBPβ binding site or responsive element within this region has yet to be explored.

As a versatile transcription factor modulating a number of cellular functions, C/EBPβ has been implied by a number of studies to be an important piece of the puzzle in the pathophysiology of GCT of bone. Interleukin-6 (IL-6) has been shown to be up-regulated in GCT and stimulates bone resorption [Ohsaki et al., 1992]. It also induced *RANKL* expression in a STAT3-dependent manner

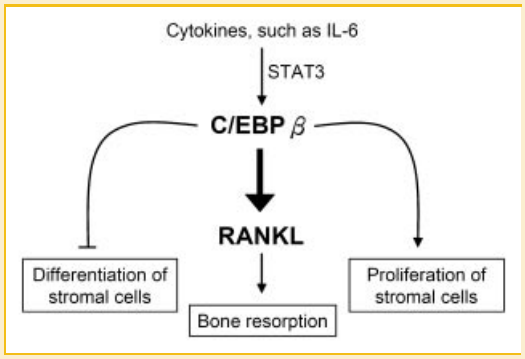

Fig. 7. Proposed signaling network of C/EBPβ in the stromal cells of GCT of bone. Lines with arrowhead indicate activation, while line with blunt-end indicates inhibition. The bold solid line indicates the findings of this study (C/EBPβ activates *RANKL* transcription); other solid lines indicate the relationships supported by the literature.

in osteoblastic and stromal cells and thus enhanced osteoclast formation [Udagawa et al., 1995; O'Brien et al., 1999]. However, how *RANKL* expression is induced by IL-6 has yet to be elucidated. Our present finding, along with that of Niehof et al. that IL-6 induced *C/EBPβ* transcription through recruitment of STAT3 to its promoter [Niehof et al., 2001], allows us to propose a model of RANKL activation. IL-6 induces *C/EBPβ* expression through STAT3 recruitment onto the *C/EBPβ* promoter and then C/EBPβ induces *RANKL* expression through binding to the CCAAT-box on the *RANKL* promoter (Fig. 7).

A putative pathological phenomenon in GCT of bone is the failure of terminal differentiation of stromal cells into mature osteoblasts. It has been further demonstrated by us and others that stromal cells in GCT of bone have an osteoblast lineage and are capable of differentiating into osteoblasts [Huang et al., 2004; Murata et al., 2005]. However, the cause of this "arrested" differentiation remains unknown. Recently, decline of *C/EBPβ* expression was reported during mid-stage differentiation of primary rat osteoblast cultures, and stable expression of C/EBPβ was found to enhance proliferation and correspondingly suppressed differentiation of osteoblasts [Mandrup and Lane, 1997]. Therefore, it is reasonable to hypothesize that the induced expression of *C/EBPβ* found in GCT stromal cells enhances proliferation and suppresses differentiation of the cells contributing to the prevention of osteoblastic differentiation of stromal cells (Fig. 7).

To conclude, we found that C/EBPβ is up-regulated in GCT stromal cells compared to the normal osteoblasts and it can induce RANKL promoter activity in dose-dependent manner. CCAAT-box located around 50-bp upstream of TSS of the *RANKL* promoter is vital for the basal transcription. In vivo binding of C/EBPβ to the *RANKL* promoter was also demonstrated in GCT stromal cells. Taken together, C/EBPβ is believed to be one of factors inducing *RANKL* expression and triggering bone over-resorption in GCT of bone.

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