

## **IkappaBalpha regulates Hes1 in osteoclast differentiation and resorption**

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## **1. ABSTRACT**

During osteoclast differentiation and resorption, both NF-kappaB and Notch signalling are activated. This study defines the mechanism about the influence of NFkappaB on Notch. To this end, IkappaBalphaM and Wild-type-IkappaBalpha were transfected into RAW 264.7 cells. The number of cells that differentiated into osteoclasts was quantified. The resorption area was measured. NF-kappaB transcriptional activity was determined by EMSA. Hes1, DC-STAMP and MMP-9 mRNA expression levels were determined by RT-PCR. Hes1 protein expression was determined by western blots. ChIP was used to study binding of IkappaBalpha to the Hes1 promoter. NF-kappaB inactivation inhibited the differentiation and resorption ability of osteoclasts. Compared with Wild-type cells, NF-kappaB inactivation resulted in an up-regulation of Hes1 expression, and a down-regulation of DC-STAMP and MMP-9 expression. Moreover, in response to RANKL, NF-kappaB inactivation resulted in a down-regulation of DC-STAMP and MMP-9 expression compared with Wild-type cells. The Hes1 promoter was detected by ChIP using IkappaBalpha antibody. In conclusion, our data suggest IkappaBalpha regulates Hes1-mediated activity in osteoclast differentiation and resorption, which support a cross-talk between NF-kappaB and Notch in osteoclast activity.

## **2. INTRODUCTION**

The development and continuous remodeling of the skeleton demand a tightly regulated balance between bone-formation and bone-resorption. Although many aspects of the resorptive processes remain elusive, a general understanding is that the osteoclast is the pivotal cell in the degradation of the bone matrix (1). Osteoclasts are derived from hematopoietic myeloid precursors of the monocyte/macrophage lineage and under the control of systemic and local factors produced by supporting cells such as osteoblasts and bone marrow stromal cells. Among these factors, RANKL is the key cytokine that stimulates osteoclast differentiation both *in vitro* and *in vivo* (2-4). RANK, the receptor of RANKL, is expressed on the surface of osteoclast progenitor cells. When RANK binds to its ligand, intracellular signals including NF-kappaB are induced, leading to osteoclast formation (5).

NF-kappaB is a transcriptional factor family. It consists of c-Rel, p50, p52, p65 and RelB, which can form various homodimers or heterodimers through a highly conserved N-terminal Rel homology domain. In the cytoplasm of quiescent cells, the dimers bind inhibitory molecules of the IkappaB family. Cell activation by various stimuli including RANKL results in serine phosphorylation and degradation of IkappaB. Subsequently, NF-kappaB

dimers translocate into the nucleus and regulate downstream gene expressions (6-8). Moreover, IkappaBalpa can also shuttle between nucleus and cytoplasm (9, 10), which adds complexity to the system. Although nucleocytoplasmic shuttling of IkappaBalpa is important for the termination of NF-kappaB signaling, its physiological effect has not been completely understood. It is possible that IkappaBalpa may have an additional unidentified function. It has been recently demonstrated that TNF-alpha stimulation led to the release of IkappaBalpa and up-regulation of Notch-dependent Hes1 (Hairy and enhancer of split) gene expression in fibroblast 3T3 cells and human embryonic kidney 293T cells (11). Hes1 encodes a basic helix-loop-helix transcription factor that functions as a classical effector of the Notch signaling pathway (12).

Recent findings suggest an important role of Notch in cell-fate determination of haematopoietic progenitor cells (13). In canonical Notch signaling, binding of transmembrane Notch receptors (N1-N4) by Jagged or Delta ligands results in gamma-secretase-mediated cleavage and release of the Notch intracellular domain, its nuclear translocation and association with the transcriptional activator Mastermind, and DNA-binding protein, CSL/RBP-J (14). This heterotrimeric complex induces the expression of Notch target genes, such as Hes1 (12).

As cells are frequently exposed to simultaneous activation of different signaling pathways, a fundamental question is, how different transduction pathways integrate into the final message resulting in a biological response? The aim of the present study was to investigate the possible coordination between the NF-kappaB and the Notch signaling pathway in osteoclast formation and function. Here, we present evidence that the regulation of Hes1 by NF-kappaB occurs via a mechanism that is dependent on the release of IkappaBalpa from the Hes1 promoter in RANKL-induced osteoclast activity. It has important implications for future therapeutic approaches directed against these signaling pathways involved in bone diseases.

### **3. MATERIALS AND METHODS**

#### **3.1. Cell culture, transfection, and infection**

RAW 264.7 and 293T cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). RAW 264.7 cells were cultured in RPMI1640 and 10% FBS. 293T cells were cultured in DMEM and 10% FBS. Cells were plated at sub-confluence. P-Retro-on-IkappaBalpaM [phosphorylation defective IkappaBalpa (S32, 36A)] and Wild-type plasmids were transfected into 293T using FuGENE 6 (Roche Applied Science, Roche GmbH, Mannheim, Germany), and virus supernatants were harvested to infect RAW 264.7 cells. Stable sub-clones of Mutant and Wild-type were selected with puromycin.

#### **3.2. Osteoclast formation assays**

Mutant and Wild-type cells were plated at a density of  $1.3 \times 10^4/\text{cm}^2$  into chamber slides (Nalge Nunc International, Rochester, New York, USA) in the presence

of RANKL (100ng/ml). The medium was changed every 2 days. After 5 days of culture, the cells were fixed and stained using the TRAP staining kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. TRAP-positive cells with more than three nuclei were considered to be osteoclast-like cells. The number of osteoclast-like cells was counted under a light microscope.

#### **3.3. Resorption assays**

Mutant and Wild-type cells were plated at a density of  $1.3 \times 10^4/\text{cm}^2$  onto Osteoclast Activity Assay Substrate (Osteogenic Core Technologies, Chunan, Choongnam, Korea) and treated with 100 ng/ml RANKL. Medium was changed every 2 days. After 10 days of culture, the plates were washed to remove the cells. The resorption areas on the plates were photographed with a digital camera attached to the microscope and analyzed by the Image Analysis System (Leica, Solms, Germany).

#### **3.4. Real-time PCR**

Mutant and Wild-type cells were plated at a density of  $1.3 \times 10^4/\text{cm}^2$  in 60 mm tissue culture dishes in the presence of RANKL (100 ng/ml). After 24 h of incubation, total RNA was extracted using Absolutely RNA Miniprep kit (Stratagene, CA, USA) according to the manufacturer's instructions. cDNA was synthesized with superscript reverse transcriptase (Invitrogen, Nelson, UK), followed by quantitative real time PCR with gene specific primers. Hes1 primers (5-ACCCAGCCAGTGTCACA-3 and 5-CATTGATAGGCTTTGATGACTTTCTG-3), DC-STAMP primers (5-TTGCCGCTGTGGACTATCTG -3 and 5-GAATGCAGCTCGGTTCAAAC -3), and MMP-9 primers (5-TTCCCCAAAGACCTGAAAACC-3 and 5-GCCCGGGTGTAACCATAGC-3) were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA). Relative quantities of mRNA expression were analysed using Quantitative RT-PCR (Bio-Rad, CA, USA) in the presence of SYBR green (Applied Biosystems, Foster City, CA, USA). For the normalization of each sample, the expression level of the gene was divided by that of beta-actin.

#### **3.5. Cell extracts preparation and Western blot analysis**

Mutant and Wild-type cells were washed twice with ice-cold PBS, scraped and collected by centrifugation. Cell pellets were lysed with cytoplasmic lysis buffer. The tubes were gently vortexed for 15 min. Nuclei were collected by centrifugation at 8000 g for 15 min, and the supernatants were stored at -80 °C. To obtain nuclear protein extracts, the pellets were resuspended in lysis buffer, and gently shaken for 30 min at 4 °C, followed by centrifugation at 13,000 g for 15 min. Protein concentrations of the extracts were determined by using the Bio-Rad protein agent (Bio-Rad, Muchen, Germany). Fifty micrograms of total protein was resolved on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, and probed with primary antibody Phospho-NF-kappaB p65 (Ser536) (Cell Signaling Technologies, Beverly, MA, USA) and Hes1 (Santa Cruz Biotech, Santa Cruz, CA, USA). Specific binding was detected using a secondary antibody coupled to horseradish peroxidase and the Lumi-light Plus

Western blot substrate (Roche diagnostics GmbH, Munnheim, Germany). After detection, blots were stripped and reprobed with primary antibody beta-actin (Santa Cruz Biotech, Santa Cruz, CA, USA). The bands were quantified by Quantity One software (Bio-Rad, CA, USA).

### 3.6. Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay (EMSA) was performed using NF-kappaB oligonucleotide probe (5'-AGTTGAGGGGACTTTCACAGGC-3') end-labeled with [ $\gamma$ - $^{32}$ P]. Nuclear protein (2  $\mu$ g) was pre-incubated in binding buffer, consisting of 10 mM Tris-Cl, pH 7.5, 1 mM MgCl<sub>2</sub>, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol, and 0.05  $\mu$ g/ml of poly (dI-dC) (deoxyinosinic deoxycytidylic acid) (Amersham Biosciences) for 15 min at room temperature. After addition of the 2  $\mu$ l [ $\gamma$ - $^{32}$ P]-labeled oligonucleotide probe, the incubation was continued for 30 min at room temperature. The reaction was terminated by adding 1  $\mu$ l gel loading buffer, and the mixture was subjected to non-denaturing 4% polyacrylamide gel electrophoresis in 0.5  $\times$  TBE buffer. The gel was exposed to X-ray film for 1 h to overnight at -80 °C with an intensifying screen and scanned. The bands were quantified by Quantity One software..

### 3.7. Chromatin immunoprecipitation (ChIP)

Approximately  $2 \times 10^6$  RAW 264.7 cells with or without RANKL stimulation were cross-linked in the presence of 1% formaldehyde for 15 min at room temperature. Cross-linking was stopped on addition of glycine to a final concentration of 125 mM for 5 min at room temperature. Cells were washed twice with cold  $1 \times$  PBS, scraped, and centrifuged at 100g for 5 min. Cell pellets were resuspended in 400  $\mu$ l lysis buffer (5 mM Pipes [KOH], pH 8.0, 85 mM KCl, 0.5% NP40, containing complete protease inhibitors) and incubated on ice for 10 min. Nuclei were pelleted in a microcentrifuge at 1500g for 5 min at 4 °C. The nuclei preparations were resuspended in 200  $\mu$ l nuclear lysis buffer (50 mM Tris, pH8, 10 mM EDTA, pH 8, 1% SDS, complete protease inhibitors) and incubated on ice for 10 min. While on ice, samples were sonicated with 6 $\times$ 20s bursts using a branson sonifier. Debris was cleared by centrifugation at 15,800g for 5 min. The supernatant was transferred to a new tube and diluted 5-fold in chromatin immunoprecipitation (ChIP) dilution buffer (0.01% SDS, 1.1% TritonX 100, 1.2 mM EDTA, 16.7 mM Tris, pH8, 167 mM NaCl including complete protease inhibitors). To reduce nonspecific background, the samples were pre-cleaned with 40  $\mu$ l protein G sepharose and 20  $\mu$ g salmon sperm DNA. Twenty percent of the samples were kept as a total input control. Anti- IkappaBalpa antibody or rabbit Ig (10  $\mu$ g/ml) was used for immunoprecipitation, prearmed with 20  $\mu$ l protein G sepharose /20  $\mu$ g salmon sperm DNA slurry overnight at 4 °C. Beads were washed three times in low salt buffer (0.1% SDS, 1.0% TritonX 100, 2 mM EDTA, 20 mM Tris, pH8, 150 mM NaCl) for 10 min at 4°C and three times with high salt buffer (0.1% SDS, 1.0% TritonX 100, 2 mM EDTA, 20 mM Tris, pH8, 500 mM NaCl). Cross-linkage was reversed by heating at 65°C, followed by treatment with 40  $\mu$ g/ml proteinase K at 45 °C for 60min. DNA was recovered by phenol-chlorophorm-ethanol precipitation and used as a template for PCR to

amplify the region including the IkappaBalpa site in the Hes1 promoter. Primers for input and sample preparations: Forward 5'- TTCCTCCCATTGGCTGAAAG-3'; Reverse 5'-GTGATCGGTAGCACTATTCC-3' (195bp, NC000082). PCR amplification was achieved at 94°C/30s; 58°C/30s and 72°C/30s for 25 cycles. The PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide.

### 3.8. Data analysis

Data were expressed as mean  $\pm$  SD. Statistical differences were determined using ANOVA or student's t test for repeated measures;  $p < 0.05$  was considered to be significant.

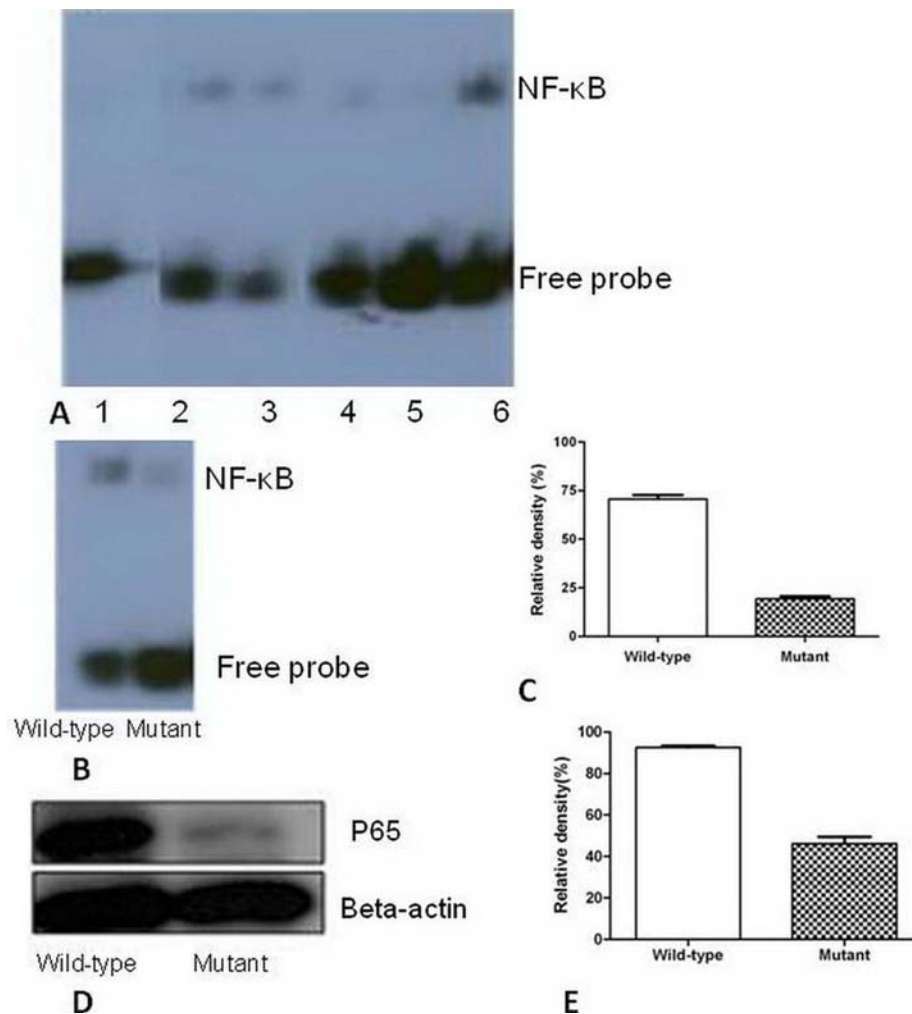
## 4. RESULTS

### 4.1. Stable expression of IkappaBalpaM in RAW 264.7 cells

To profile the extent of IkappaBalpaM expression in RAW 264.7 cells used for the present studies, we selected several subclones stably expressing exogenous IkappaBalpaM (Mutant cells), as well as several RAW 264.7 subclones stably expressing the IkappaBalpa gene (Wild-type cells), transfected with IkappaBalpa vectors, as a control. NF-kappaB DNA binding activity was detected with EMSA. First, the NF-kappaB probe specificity was tested with competitive analysis (Figure 1A). Shift bands decreased with increasing amount of unlabelled NF-kappaB while no change of shift band with addition of noncompetitive SP1 probe. EMSA results show that RANKL increased amounts of nuclear complex protein of NF-kappaB in Wild-type cells compared with Mutant cells (Figure 1B, 1C). A 2.1-fold decrease in the level of p65 nuclear protein was observed in Mutant cells (Figure 1D, 1E), whereas positive, but relatively higher level, of p65 nuclear protein was expressed in control cells (Wild-type cells). EMSA and Western blot results demonstrated that NF-kappaB signaling has been inactivated after IkappaBalpaM vector transfected into RAW 264.7 cells.

### 4.2. NF-kappaB mediates RANKL-induced differentiation and resorption

Osteoclasts are multinucleated cells with TRAP-positive staining. TRAP staining showed that osteoclast number increased after treatment with RANKL for 5 days. Osteoclast formation was impaired in Mutant cells compared with Wild-type cells (Figure 2A). These results clearly indicate a role of NF-kappaB in osteoclast differentiation. Bone resorption assays were subsequently carried out for understanding whether the osteoclast-like cells induced by RANKL are active. As shown in the Figure 2C, some pit formation was detectable in the cultures of Mutant cells. However, the number and the size of pits formed were further augmented in the cultures of Wild-type cells when these cells were incubated at the same conditions (Figure 2B). Resorption assay results showed the resorption activity of RAW 264.7 cells induced by RANKL was significantly inhibited after NF-kappaB inactivation (Figure 2D).



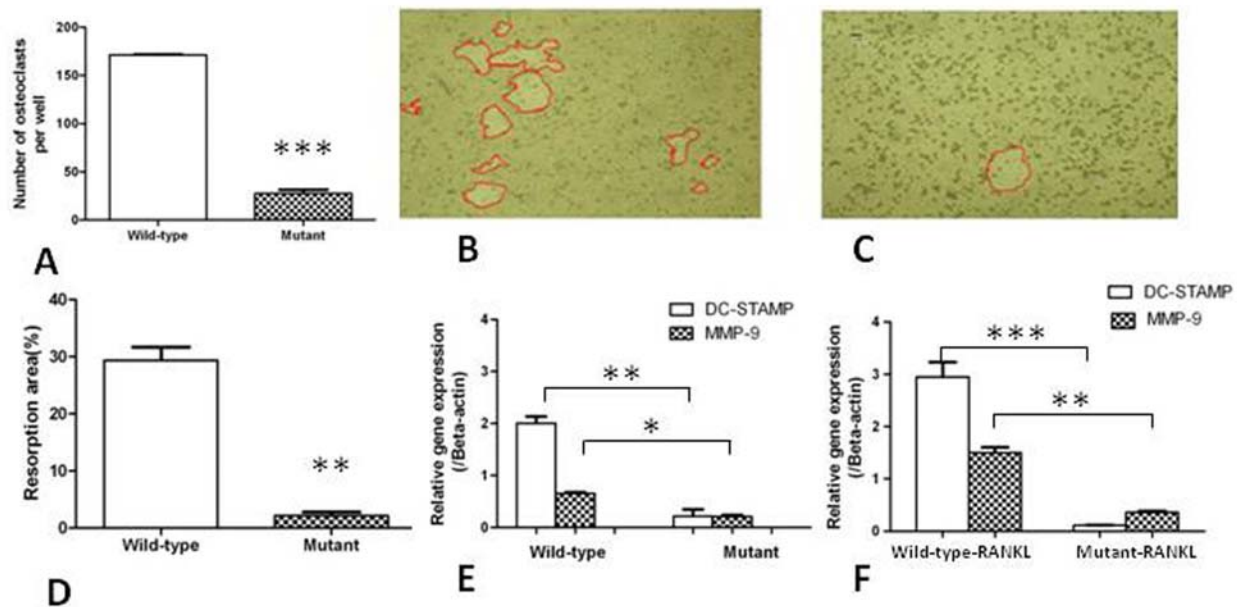
**Figure 1.** NF- $\kappa$ B signaling was inactivated by stable transfection of IkappaBalphaM. RAW 264.7 cells were infected by virus supernatant from 293T transfected with IkappaBalphaM/IkappaBalpha. Stable sub-clones of Mutant and Wild-type cells were selected with puromycin. (A) Competitive analysis. Nuclear extracts were prepared from RAW 264.7 cells and subjected to analysis for NF- $\kappa$ B DNA binding activity, as measured by EMSA. (1) without nuclear extracts; (2) nuclear extracts and [ $\gamma$ - $^{32}$ P]-labeled NF- $\kappa$ B Oligo; (3) nuclear extracts and [ $\gamma$ - $^{32}$ P]-labeled NF- $\kappa$ B Oligo in the presence of 50 $\times$  unlabeled NF- $\kappa$ B Oligo; (4) nuclear extracts and [ $\gamma$ - $^{32}$ P]-labeled NF- $\kappa$ B Oligo in the presence of 100 $\times$  unlabeled NF- $\kappa$ B Oligo; (5) nuclear extracts and [ $\gamma$ - $^{32}$ P]-labeled NF- $\kappa$ B Oligo in the presence of 200 $\times$  unlabeled NF- $\kappa$ B Oligo; (6) nuclear extracts and [ $\gamma$ - $^{32}$ P]-labeled NF- $\kappa$ B Oligo in the presence of 200 $\times$  unlabeled SP1 probe. (B) Nuclear extracts were prepared from Wild-type and Mutant cells after RANKL treatment. The DNA binding activity was measured. (C) The histogram indicates the relative band intensity of DNA-protein shift. Values are derived from densitometric scans of 3 independent experiments. Results are expressed as percentage of NF- $\kappa$ B shift band density to that of free probe. (D) Western blot result of nuclear protein for p65 is shown. (E) The histogram indicates the relative band intensity of Western blot. Values are derived from densitometric scans of 3 independent experiments. A representative experiment for Western blot and EMSA is shown of three independent experiments performed with similar results.

#### 4.3. NF- $\kappa$ B inactivation down-regulates MMP-9 and DC-STAMP expression

MMP-9 and DC-STAMP are target genes of NF- $\kappa$ B signaling. In the absence of stimuli, RT-PCR results demonstrated that MMP-9 and DC-STAMP expression was down-regulated after NF- $\kappa$ B inactivation (Figure 2E). In response to RANKL, RT-PCR results demonstrated that MMP-9 and DC-STAMP expression was down-regulated after NF- $\kappa$ B inactivation (Figure 2F).

#### 4.4. NF- $\kappa$ B inactivation up-regulates Notch-dependent Hes1 gene expression

Expression of NF- $\kappa$ B M in RAW 264.7 cells results in NF- $\kappa$ B inactivation. In the absence of stimuli, we detected higher levels of Hes1 mRNA and protein in Mutant cells compared with Wild-type cells (Figure 3A, 3B and 3C). Furthermore, Hes1 mRNA and protein expression was up-regulated after RANKL treatment in Wild-type cells (Figure 3A, 3D and 3E).



**Figure 2.** Effects of NF-kappaB inactivation on the differentiation and resorption of RAW 264.7 cells induced by RANKL. Osteoclasts are multinucleated cells with TRAP-positive staining. For differentiation assay, Mutant and Wild-type cells were cultured with RANKL stimulation for 5 days. (A) The number of osteoclasts was counted under light microscope. The data are the mean  $\pm$  SD for the cultures. For resorption assay, cells were seeded on Osteoclast Activity Assay Substrate plates. Treatment with RANKL for 10 days, pit formation was analyzed under microscope. The red mark indicates the resorption area. (B) Wild-type. Magnification 5.0 $\times$ 3.6. (C) Mutant. Magnification 5.0 $\times$ 3.6. (D) Percentage of resorption area per well was measured by image analyzer and plotted. Values shown are mean  $\pm$  SD of three independent experiments with triplicate cultures. (E) Cells were harvested and the expressions of MMP-9 and DC-STAMP mRNA were examined with real-time PCR. (F) Cells were treated with RANKL for 24h. Then cells were harvested and the expressions of MMP-9 and DC-STAMP mRNA were examined with real-time PCR. The results were expressed as the ratio of gene expression to beta-actin. Values presented are the mean  $\pm$  SD of three replicate measurements. Statistical analysis was performed by student's t-test. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001

#### 4.5. NF-kappaB inactivation exerts a positive effect on the transcriptional activation of the Hes1 promoter

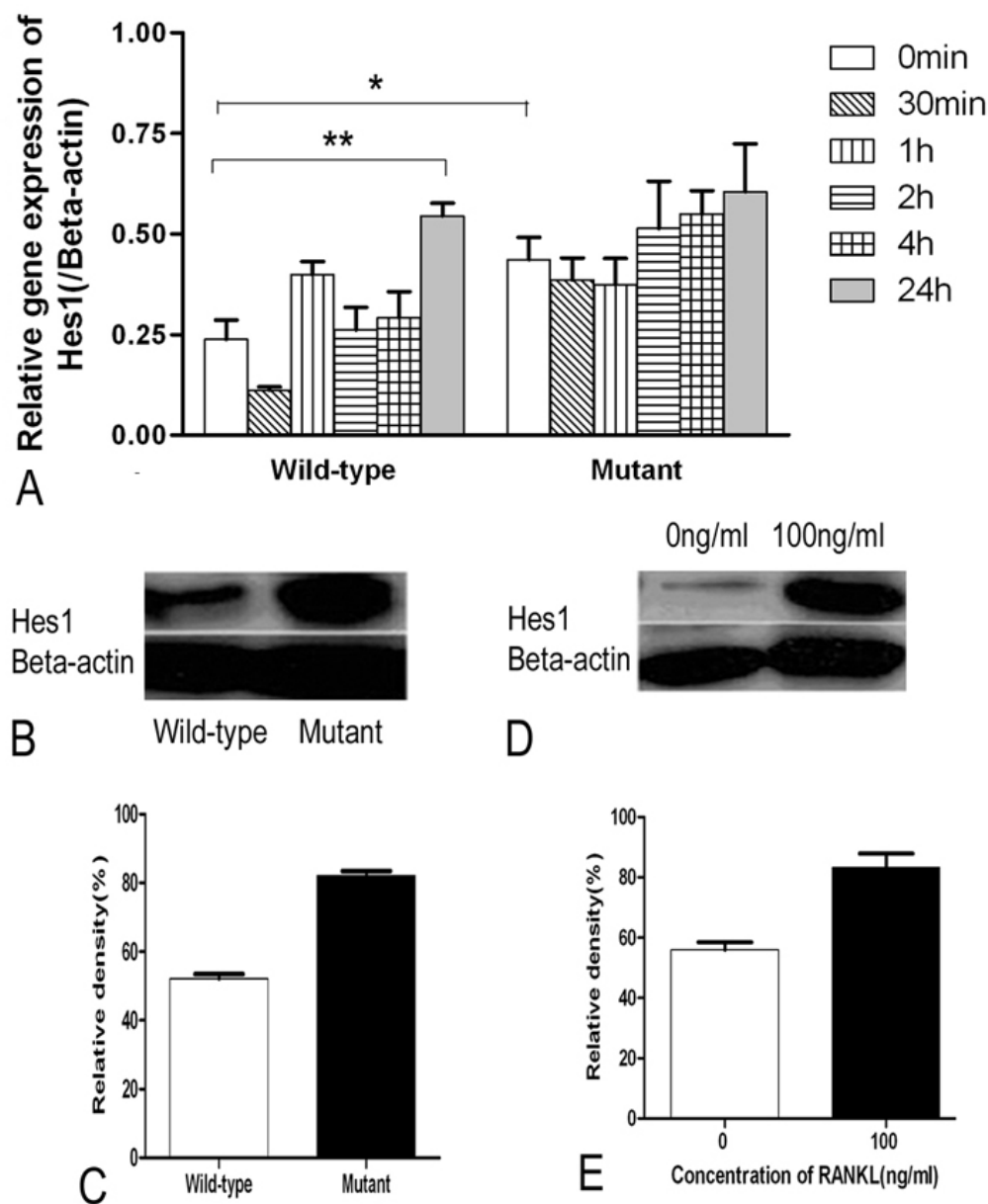
As IkappaBalpha modulates the Hes1 gene expression, we hypothesized that the IkappaBalpha fraction interacts with the repressor elements was directly responsible for Hes1 expression. To test this, ChIP assays were performed to investigate whether IkappaBalpha protein was combined to the promoter region of this gene. Hes1 promoter was detected from chromatin precipitated with IkappaBalpha antibody, thus indicating that this promoter is indeed able to bind IkappaBalpha. As a control, Hes1 promoter could not be amplified from precipitates with nonrelevant immunoglobulins or with RANKL treatment (Figure 4A, 4B). These observations strongly support that chromatin-associated IkappaBalpha is involved in the recruitment of specific repression complexes in the noninduced state, and RANKL regulates Hes1 transcriptional activation by modulating the association of IkappaBalpha with the Hes1 promoter.

## 5. DISCUSSION

It is well established that RANKL regulates osteoclast differentiation and resorption mainly via NF-kappaB signaling (15). Our results further confirmed that

NF-kappaB signaling is involved in RANKL-induced osteoclast differentiation and resorption. In these processes, NF-kappaB regulates Notch signaling. This is the first study reporting that NF-kappaB influences Notch via the binding of Hes1 promoter in osteoclast activity.

It is generally accepted that NF-kappaB plays an important role in osteoclast formation. TRAP staining is an often-used method to examine osteoclast formation, and TRAP-positive multinucleated cells including osteoclasts or osteoclast-like cells (16, 17). In this study, TRAP-positive multinucleated cells were observed after RANKL-induced NF-kappaB activation, whereas the number of RANKL-induced TRAP-positive multinucleated cells was significantly decreased, after NF-kappaB inactivation by IkappaBalphaM transfection. Studies have demonstrated that RANKL modulates the DC-STAMP expression in osteoclast formation (18) and NF-kappaB binds directly to the DC-STAMP promoter (19). Moreover, MMP-9 plays a crucial role in bone matrix degradation (20) and MMP-9 expression is modulated by RANKL through regulation of NF-kappaB followed by NFAT activation (18). Our study shows that NF-kappaB inactivation results in decreased DC-STAMP and MMP-9 expression as well as bone resorption ability of osteoclasts, thus providing further

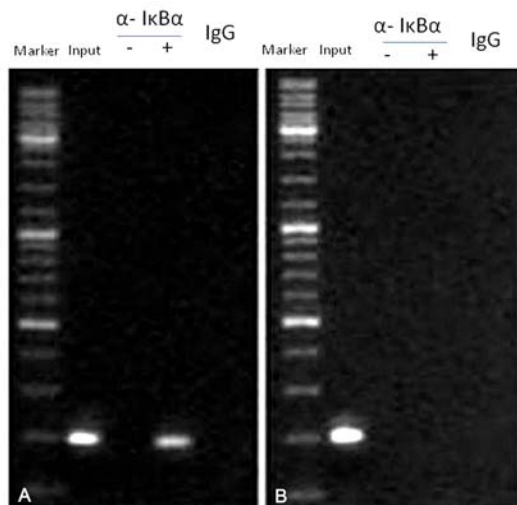


**Figure 3.** Effects of NF-kappaB inactivation on the Hes1 expression of RAW 264.7 cells. (A) With RANKL treatment for varying time intervals, cells were harvested and the expression of Hes1 mRNA was examined with real-time PCR. The results were expressed as the ratio of Hes1 to beta-actin. (B) Total protein from cells without RANKL stimulation was harvested, Western blot result of Hes1 is shown. (C) The intensity of the Western blot bands was determined by densitometry and normalized with that of beta-actin. (D) Without or with RANKL stimulation for 48 h, whole cell lysates of Wild-type cells were prepared, and equal amount of proteins (15µg) were analyzed by Western blot using antibody to Hes1. (E) The intensity of the Western blot bands was determined by densitometry and normalized with that of beta-actin. Values shown are mean  $\pm$  SD of three independent experiments. Statistical analysis was performed by student's *t*-test or ANOVA. \**P*<0.05, \*\**P*<0.01.

evidence that NF-kappaB plays a role in osteoclast formation and resorption.

Notch and NF-kappaB pathways are involved in the regulation of gene expression and cellular events in many different systems. The effect of each pathway in inhibiting or promoting differentiation is controversial and

it could depend on the cellular context (21-23). Furthermore, Notch and NF-kappaB exert antagonistic or synergistic effects in different system (24, 25). However, the results of this study strongly support the view that NF-kappaB promotes osteoclast differentiation in response to RANKL stimulation. Meanwhile, Notch-dependent Hes1 is up-regulated and the Notch pathway is activated.



**Figure 4.** Hes1 promoter is occupied by IkappaBalpa in RAW 264.7 cells. (A) Chromatin obtained from RAW 264.7 cells was immunoprecipitated with IkappaBalpa antibodies or irrelevant immunoglobulins. Recruitment of IkappaBalpa to the Hes1 promoter was analyzed by PCR. (B) Chromatin obtained from RAW 264.7 cells with RANKL treatment was immunoprecipitated with IkappaBalpa antibodies or irrelevant immunoglobulins. Recruitment of IkappaBalpa to the Hes1 promoter was analyzed by PCR.

Our results demonstrated that recruitment of IkappaBalpa to the Hes1 promoter mediated the process in which NF-kappaB regulates Notch activity. RANKL treatment results in the release of Chromatin-associated IkappaBalpa from the Hes1 promoter, coinciding with the transcriptional activation of this gene. This finding suggested a cross-talk between the NF-kappaB and Notch pathways exists in osteoclasts. Such cross-talk may contribute to additional complexity in the regulation of cell behavior. In the immune system, NF-kappaB mediates Jagged1 expression, which can initiate signaling downstream of Notch (26). However, in the 32D myeloid progenitor system, NF-kappaB inhibits Notch-dependent gene Hes1 (27). On the other hand, many cellular processes have been reported in which activation of Notch signaling correlates with the inhibition of the NF-kappaB pathway. Whether Notch activation enhances or inhibits the activity of NF-kappaB in RAW 264.7 cells in response to RANKL awaits confirmation in our future study.

In conclusion, this study showed that recruitment of IkappaBalpa to the Hes1 promoter was associated with transcriptional repression, and this mechanism was involved in the osteoclast differentiation and resorption. Our findings may provide important clues to understanding the coordination of Notch and NF-kappaB pathways in controlling osteoclast cellular events, and to modulating the state of bone metabolic diseases.

## 6. ACKNOWLEDGMENTS

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**Abbreviations:** NF- $\kappa$ B: nuclear factor  $\kappa$  B; RANKL: nuclear factor  $\kappa$  B ligand; IkappaB $\alpha$ : nuclear factor of  $\kappa$  light polypeptide gene enhancer in B-cells inhibitor,  $\alpha$ ; Hes1: hairy and enhancer of split; DC-STAMP: dendritic cell-specific transmembrane protein; MMP-9: matrix metalloproteinase-9; CSL: Delta/Serrate/LAG-2; RBP-J: recombining binding protein suppressor of hairless; EMSA: electrophoretic mobility shift assay; ChIP: chromatin immunoprecipitation; RT-PCR: real-time polymerase chain reaction; TRAP: tartrate resistant acid phosphatase

**Key Words:** Osteoclast, IkappaB $\alpha$ , Hes1, NF- $\kappa$ B, Notch

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