Molecules and Cells

ST5 Positively Regulates Osteoclastogenesis via Src/Syk/Calcium Signaling Pathways

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For physiological or pathological understanding of bone disease caused by abnormal behavior of osteoclasts (OCs), functional studies of molecules that regulate the generation and action of OCs are required. In a microarray approach, we found the suppression of tumorigenicity 5 (ST5) gene is upregulated by receptor activator of nuclear factor-κB ligand (RANKL), the OC differentiation factor. Although the roles of ST5 in cancer and β-cells have been reported, the function of ST5 in bone cells has not yet been investigated. Knockdown of ST5 by siRNA reduced OC differentiation from primary precursors. Moreover, ST5 downregulation decreased expression of NFATc1, a key transcription factor for osteoclastogenesis. In contrast, overexpression of ST5 resulted in the opposite phenotype of ST5 knockdown. In immunocytochemistry experiments, the ST5 protein is co-localized with Src in RANKL-committed cells. In addition, ST5 enhanced activation of Src and Syk, a Src substrate, in response to RANKL. ST5 reduction caused a decrease in RANKL-evoked calcium oscillation and inhibited translocation of NFATc1 into the nucleus. Taken together, these findings provide the first evidence of ST5 involvement in positive regulation of osteoclastogenesis via Src/Syk/calcium signaling.

Keywords: calcium, NFATc1, osteoclasts, RANKL, Src, suppression of tumorigenicity 5, Syk

INTRODUCTION

Bone is a dynamic organ that is constantly being disrupted and restructured for maintenance of homeostasis (Zaidi, 2007). The process of bone resorption is controlled by action of osteoclasts (OCs), which are multinucleated cells (MNCs) generated from a monocyte/macrophage lineage of hemopoietic cells (Boyle et al., 2003; Rho et al., 2004). The action of excessive OCs induces an imbalance in homeostasis, triggering pathological bone diseases, such as osteoporosis (Zaidi, 2007).

Osteoclastogenesis is a multi-step process including commitment of OC precursor cells to mononucleated preosteoclasts (pOCs), fusion for formation of multinucleated OCs, and maturation of OCs for bone resorption activity. The process requires the action of two essential factors, macrophage-colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) (Boyle et al., 2003; Rho et al., 2004). The binding of RANKL to its receptor (RANK) initiates recruitment of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (Darnay et al., 1999) and activates downstream signaling molecules including MAPKs (ERK, JNK, and p38), NFκB, and AP-1 complex (a protein complex of c-Jun and c-Fos) (Park et al., 2017; Takayanagi, 2005). Finally, these signaling pathways induce expression of the nuclear factor of activated T cells c1 (NFATc1), which is a key transcription factor for osteoclastogenesis. NFATc1

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induces OC-specific genes, including tartrate-resistant acid phosphatase (TRAP) and cathepsin K (Kim and Kim, 2014). Calcium signaling is known to play a role in NFATc1 activation by promoting its nuclear translocation. When RANKL triggers a signal for induction of osteoclastogenesis, calcium signaling is stimulated via activation of PLCγ, which leads to generation of inositol 1,4,5-trisphosphate (IP3) and its binding to IP3 receptors on the endoplasmic reticulum (ER) (Kim and Kim, 2014; Koga et al., 2004). In addition, Src- and Src substrate Syk-dependent signaling is involved in activation of PLCγ by RANKL (Tomomura et al., 2012; Yamasaki et al., 2014).

Suppression of tumorigenicity 5 (ST5) can exist in three isoforms including p70 (isoform3), p82 (isoform2), and p126 (isoform1) encoded by alternatively spliced transcripts (2.8 kb, 3.1 kb, 4.6 kb mRNA, respectively) (Lichy et al., 1992). The shortest form ST5 p70 is related with low tumorigenicity and is the reason for the naming of the ST5 gene (Lichy et al., 1992), whereas the longest ST5 p126 activates ERK signaling in response to EGF on COS-7 cells (Majidi et al., 1998). Physiologically, the N-terminal domain of p126 binds the Src-homology 3 (SH3) domain of c-Abl, leading to activation of the MAPK/ERK2 kinase. Moreover, the N-terminal domain of ST5 p126 also binds the SH3 domain of Src (Majidi et al., 1998). The C-terminal region of ST5 p126 binds and catalyzes the exchange of GDP to GTP of the Rab 13 protein, which promotes metastatic behavior (Ioannou et al., 2015). In addition to its function in cancer, the downregulation of ST5 has been correlated with mutation of hepatocyte nuclear factor-4α (HNF-4α), a key regulator of β-cell proliferation (Ou et al., 2018). However, to our knowledge, there is no report of ST5 expression or function in the skeletal system.

In this report, we found that ST5 is induced by RANKL during OC differentiation. In loss- and gain-of-function studies, we revealed that ST5 promotes OC differentiation by up-regulating NFATc1 via activation of Src/Syk/calcium signaling.

MATERIALS AND METHODS

Reagents

Recombinant human M-CSF and RANKL were purchased from PeproTech (USA). Anti-NFATc1 was acquired from BD Biosciences (USA). Anti-c-Fos anti-α-tubulin were purchased from Santa Cruz Biotechnology (USA). Polyclonal antibodies against ERK, JNK, p38, IκB, phospho-ERK, phospho-JNK, phospho-p38, phospho-IκB, phospho-Src family, and phospho-Syk were purchased from Cell Signaling Technology (USA). A monoclonal antibody against β-actin was obtained from Sigma Aldrich (USA). The NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit was obtained from Invitrogen (Carlsbad, CA, USA). The TRAP staining kit was purchased from Sigma Aldrich.

Animals

The ICR mice used for preparation of OC precursor cells were purchased from OrientBio (Korea). All animal experiments were performed with approval of the Institutional Animal Care and Use Committee at Seoul National University (SNU-160105-3-5) and conducted according to the Guidelines for Animal Experimentation.

Bone marrow-derived macrophage (BMM) isolation and OC differentiation

Bone marrow from the tibiae and femurs of 5-week-old ICR mice was flushed with α-MEM (Welgene, Korea). After removing erythrocytes with red blood cell lysis buffer (Invitrogen), cells were cultured in α-MEM containing 10% fetal bovine serum overnight in culture dishes. Non-adherent bone marrow cells were collected and cultured in the presence of M-CSF (30 ng/ml) for three days in 100 mm-Petri dishes. Adherent cells (BMMs) were considered to be OC precursors and harvested. BMMs were seeded at 3.5×10^4 per well in 48-well plates or 3.5×10^5 per well in 6-well plates with M-CSF (30 ng/ml) and cultured with M-CSF (30 ng/ml) and RANKL (150 ng/ml) for 4 or 5 days to induce OC formation. Cells were fixed in 3.7% formaldehyde and permeabilized using 0.1% Triton X-100. TRAP staining was performed in the dark for 10 minutes according to the manufacturer's protocol. Multinucleated TRAP-positive cells were captured and counted under an Olympus BX51 microscope (Japan).

Microarray

BMMs were cultured in α-MEM with M-CSF (30 ng/ml) and RANKL (150 ng/ml). Total RNA was prepared from untreated cells (day 0), and cells treated with RANKL (day 3) and was then hybridized with Mouse WG-6 v2.0 BeadChips (Illumina, USA). Microarray data were analyzed with the Genome Studio Gene Expression Module (Gx) v3.2 (Illumina).

ST5 gene knockdown by siRNA

BMMs were transfected with 25 nM ST5 siRNA (sc-153863; Santa Cruz Biotechnology). In brief, siRNA and transfection reagent HiPerFect (Qiagen, The Netherlands) were mixed with serum-free α-MEM medium. After the mixtures were incubated for 20 min at room temperature, they were added to the cells.

Bone resorption assay

To determine osteoclast lytic activity, BMMs (1×10^4) were seeded on dentine discs (Osteosite Dentine Discs; Immunodiagnostic Systems, UK) in 96-well plate and transfected with ST5 siRNA or control siRNA. Cells were cultured with M-CSF (30 ng/ml) and RANKL (150 ng/ml) for 7 to 9 days. The plates were rinsed with distilled water to remove cells for 15 min, and the dentine discs were carefully wiped with a cotton swab. The discs were scanned with a Carl Zeiss LSM 5-PASCAL laser-scanning microscope (Carl Zeiss Microimaging, Germany). The areas and depths of 50 resorption pits in each sample were determined with image analysis software (LSM 5 Image Browser; Carl Zeiss Microimaging). The assay was performed in triplicate wells per group.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

After extraction of total RNA using Trizol (Invitrogen), 3 μg of RNA was reverse transcribed into cDNA using Superscript II (Invitrogen) according to the manufacturer's instructions. The PCR reaction was performed as follows: denaturation at 95°C for 15 s and annealing at 60°C or 57°C for 30 s. PCR products were loaded onto 1.5% agarose gels. The *Hprt* gene

was used as a loading control. Real-time PCR was performed with a KAPA SYBR FAST qPCR kit (Kapa Biosystems, USA) in an ABI 7500 real-time system (Applied Biosystems, UK) using the following PCR conditions: 40 cycles of 3 s denaturation at 95°C and 33 s amplification at 60°C. The mRNA expression levels of genes were normalized to the mRNA expression level of the *Hprt* gene. The following PCR primer sequences were used: *St5*, 5′-tctttgcctcattgctgttg-3′ (sense) and 5′-accatcagtgcctcttc-cac-3′ (antisense); *c-fos*, 5′-ACTTCTTGTTTCCGGC-3′ (sense) and 5′-AGCTTCAGGGTAGGTG-3′ (antisense); *Nfatc1*, 5′-CCAGTATACCAGCTCTGCCA-3′ (sense) and 5′-GTGGGAAGT-CAGAAGTGGGT-3′ (antisense); *Acp5*, 5′-CGACCATTGT-TAGCCACATACG-3′ (sense) and 5′-TCGTCCTGAAGATACTG-CAGGTT-3′ (antisense); *Hprt*, 5′-CCTAAGATGAGCGCAAGTT-GAA-3′ (sense) and 5′-CCACAGGGACTAGAACACCTGCTAA-3′ (antisense).

Western blotting

Cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, proteinase inhibitor cocktail, 0.5 mM PMSF, 1 mM NaF, and 1 mM Na_3VO_4). Following determination of protein concentration with a DC Protein Assay Kit (Bio-Rad Laboratories, USA), equal amounts of lysates were loaded onto 10% SDS-polyacrylamide gels. After transferring onto nitrocellulose membranes (Amersham Pharmacia, Sweden), the membranes were blocked with 5% skim milk for 1 h and were then incubated with primary antibodies overnight at 4°C. Antibodies against NFATc1, c-Fos, α-tubulin, ERK, JNK, p38, IκB, phospho-ERK, phospho-JNK, phospho-p38, phospho-IκB, phospho-Src family, and phospho-Syk were at 1:1,000 dilution and anti-β-actin antibody was used at 1:10,000 dilution. The next day, the membranes were incubated with HRP-conjugated secondary antibodies in 2% skim milk for 1 h, and immunoreactive bands were detected using enhanced chemiluminescence reagents in a dark room. Anti-β-actin blots reprobed on the same membranes were used as loading controls.

Immunocytochemistry

To detect co-localization of ST5 and Src proteins, cells cultured onto cover glasses were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100. Cells were blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and then incubated overnight with anti-HA (1:500) antibody and anti-Src antibody (1:200) at 4°C. After incubation with Cy3 (1:200) and FITC-conjugated secondary antibody (1:200) in the dark, cover glasses were mounted with a DAPI mounting solution (Vector Laboratories, USA) and observed under a confocal microscope LSM700 (Carl Zeiss, Germany). The overlapping coefficient values of cells co-labeled with anti-HA and anti-Src were analyzed by Zen 2009 software (Carl Zeiss).

Measurement of intracellular calcium concentrations and oscillation

BMMs cultured onto cover glasses were incubated with M-CSF 30 ng/ml and RANKL 150 ng/ml for two days. Cells were loaded with Fluo-4 AM 5 μM (Invitrogen, Waltham, MA, USA) and 0.1% pluronic F127 (Sigma-Aldrich, USA) at 37°C for 30 min and then detected with an excitation/emission filter pair (485/535 nm) by a confocal microscope, with which 200 images were captured over 10 min per sample.

Extraction of cytoplasmic and nuclear proteins

Cells were washed with cold PBS twice and then fractionated with a NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. After nuclear and cytoplasmic extraction, the extracts were subjected to Western blotting. The equal protein loading of nuclear and cytoplasmic fraction was verified by blotting with anti-lamin B antibody and anti-α-tubulin antibody.

Statistical analysis

All quantitative data are presented as mean ± SD. The significance of differences between two groups was determined by Student's *t*-test. A *P* value less than 0.05 was considered significant. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, USA).

RESULTS

The level of ST5 is increased during RANKL-induced OC differentiation

In a microarray analysis performed with mouse BMMs as OC precursors cultured in the presence or absence of RANKL, we found *osteoclast associated receptor (Oscar), CD200 antigen (Cd200), St5 (isoform 1), Src, caveolin 1 (Cav1),* and *sphin-*

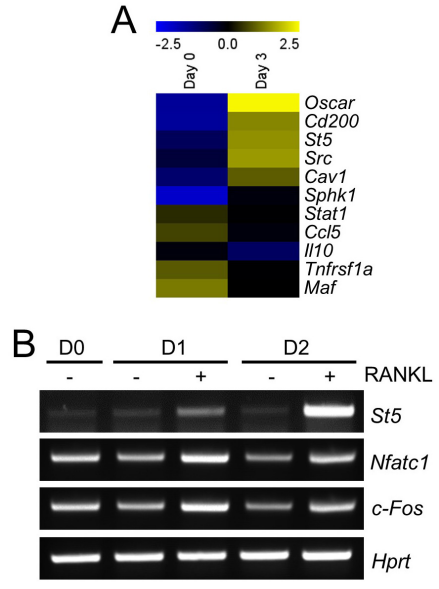

Fig. 1. Induction of ST5 positively regulates osteoclast differentiation. (A and B) BMMs were cultured with M-CSF (30 ng/ml) and RANKL (150 ng/ml) for 2 days. (A) Heatmap analysis of ST5 expression detected by microarray analysis. Yellow, upregulation; blue, downregulation. (B) The levels of *St5*, *c-Fos*, and *Nfatc1* were analyzed by RT-PCR.

gosine kinase 1 (Sphk1) among genes with increased expression during osteoclastogenesis (Fig. 1A). The genes downregulated by RANKL included *signal transducer and activator of transcription 1* (*Stat1*), *chemokine* (*C-C motif*) *ligand 5* (*Ccl5*), *TNF receptor superfamily, member 1a* (*Tnfrsf1a*), and *Maf* (Fig. 1A). Although the roles of many of the genes in the

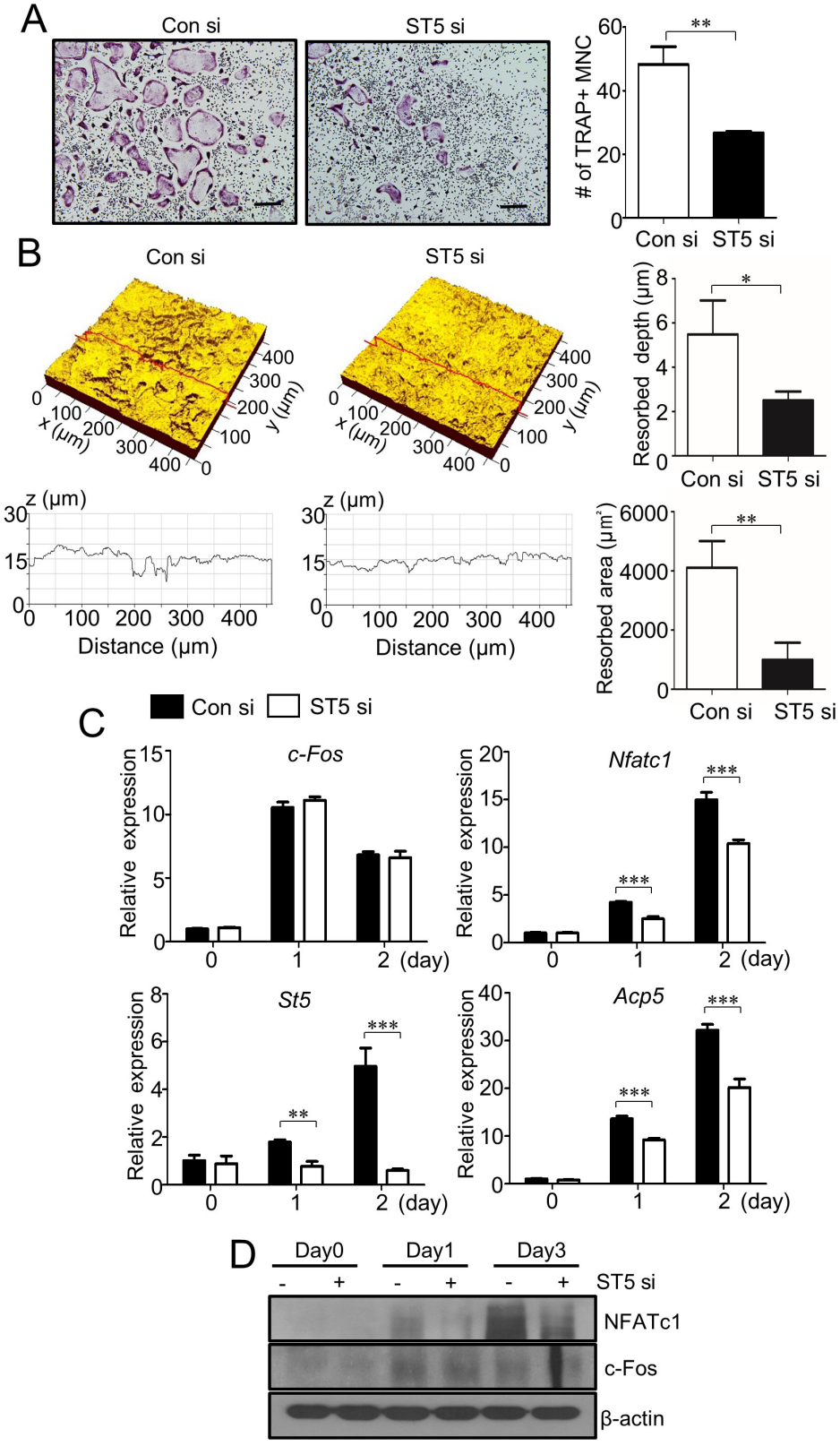

Fig. 2. Knockdown of the *St5* gene decreases RANKL-induced osteoclast differentiation. (A-D) BMMs were transfected with control siRNA or ST5 siRNA, and cells were stimulated with a medium containing M-CSF (30 ng/ml) and RANKL (150 ng/ml). (A) When MNCs were formed at day 4, cells were stained for TRAP activity. The images were captured by a light microscope, and TRAP-positive MNCs (≥ 3 nuclei) were counted as osteoclasts. Scale bars = 200 μm. (B) To examine the resorptive activity of osteoclasts, transfected BMMs were treated with M-CSF and RANKL on dentine discs for 7 to 9 days. Dentine discs were analyzed with a confocal microscope. Representative images of dentine surfaces (left), and values of resorbed depth and area of resorptive pits are presented (right). (C) The mRNA expression levels of *St5*, *c-Fos*, *Nfatc1*, and *Acp5* were analyzed by real-time PCR. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (by *t*-test). (D) The levels of c-Fos and NFATc1 expression were detected by Western blotting.

list of OC differentiation have been described (Barrow et al., 2011; Destaing et al., 2008; Evans and Fox, 2007; Kim et al., 2002; 2007; Lee et al., 2015a; 2015b; Miyamoto et al., 2012; Ryu et al., 2006; Schwartzberg et al., 1997; Takayanagi et al., 2000; Varin et al., 2013; Wintges et al., 2013), the expression or function of ST5 in OCs has not been reported. Therefore, we focused on the role of ST5 in OC differentiation. To confirm the validity of the microarray data, we examined the level of *St5* mRNA expression during OC differentiation by performing RT-PCR. As expected, expression of *c-Fos* and *NFATc1*, transcriptional factors of OC differentiation (Park et al., 2017; Takayanagi et al., 2002), was induced by RANKL, and the level of *St5* mRNA expression was increased (Fig. 1B).

Knockdown of ST5 decreases RANKL-induced osteoclastogenesis

Next, to examine the functions of ST5 in OC differentiation, we downregulated *St5* gene expression by applying the siRNA system. BMMs transfected with control siRNA or ST5 siRNA were cultured with a medium containing RANKL for 4 days, and then TRAP-positive MNCs were formed. When cells were stained for the OC differentiation marker TRAP, ST5 knockdown decreased the number of TRAP-positive MNCs compared with control (Fig. 2A). In addition, ST5 downregulated cells cultured with RANKL on dentin discs revealed diminished resorptive activity accompanying decreased resorbed depth and area versus the control group (Fig. 2B). NFATc1 is a crucial transcriptional factor which regulates the expression of essential genes such as *Acp5*, a gene encoding TRAP for OC differentiation (Hayman, 2008). The mRNA levels of *Nfatc1* and *Acp5* by ST5 siRNA were significantly decreased at 24 and 48 h after RANKL stimulation (Fig. 2C). The protein level of NFATc1 in the ST5 knockdown group was also reduced compared with the control group (Fig. 2D). On the other hand, there was no difference in levels of c-Fos mRNA and protein expression between the two groups (Figs. 2C and 2D).

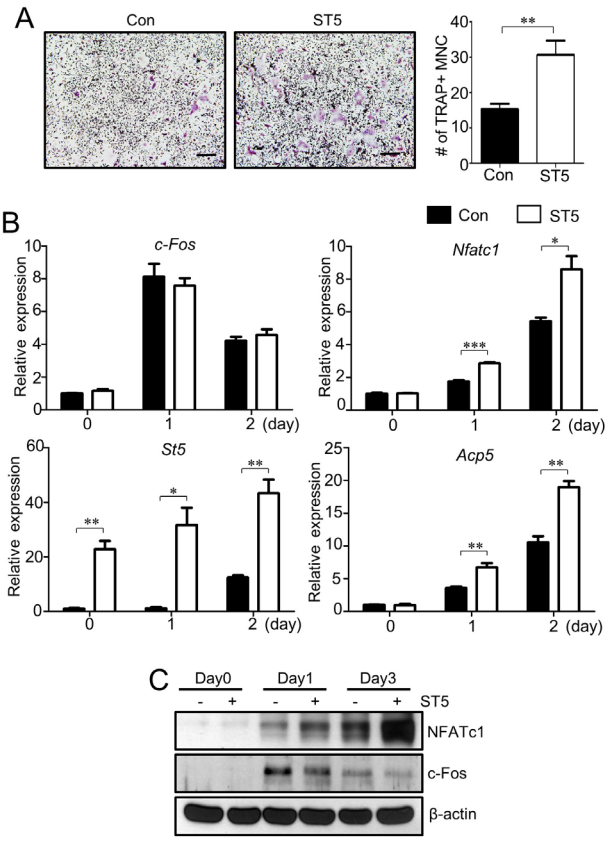

Fig. 3. Overexpression of ST5 enhances osteoclast differentiation. (A-C) BMMs transfected with ST5 expression vector were treated with RANKL (150 ng/ml) in the presence of M-CSF (30 ng/ml). (A) After MNCs were formed at day 5, cells were stained to detect TRAP activity, and TRAP+ MNCs were counted (≥ 3 nuclei). Scale bars = 200 μm. (B) The levels of *St5*, *c-Fos*, *Nfatc1*, and *Acp5* mRNA were detected. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (by *t*-test). (C) c-Fos and NFATc1 protein expression was analyzed.

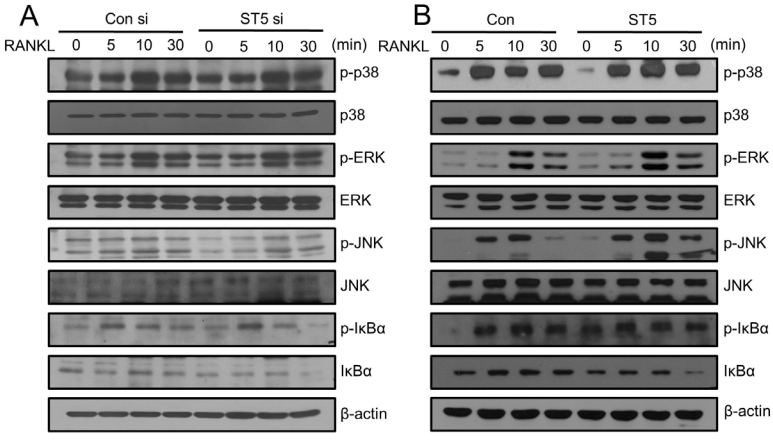

Fig. 4. ST5 does not affect NFκB and MAPKs signaling pathways. (A and B) BMMs transfected with ST5 siRNA (A) or ST5 expression vector (B) were starved for 5 h and then stimulated with serum-free medium containing RANKL (500 ng/ml) for the indicated time points, and then protein lysates were prepared. Western blotting was performed to detect the phospho-forms and total forms of the MAPKs (p38, ERK, and JNK) and IκB. β-actin is the loading control.

ST5 overexpression promotes OC differentiation by RANKL

To examine the gain-of-function effects of ST5 in osteoclastogenesis, BMMs were transfected with a *St5* gene expression vector and then cultured with RANKL. ST5 overexpression increased TRAP staining and TRAP-positive MNCs compared with the control (Fig. 3A). In line with these results, ectopic ST5 overexpression significantly increased the mRNA levels of *Nfatc1* and *Acp5* at 24 and 48 h after culturing with RANKL (Fig. 3B). Consistent with the siRNA results, we found no difference between control and ST5 overexpression in analyses of c-Fos mRNA and protein (Fig. 3C). Based on the results of knockdown and overexpression, we suggest that ST5 is a positive regulator of OC differentiation by RANKL.

ST5 does not affect RANKL-induced MAPKs and NFκB signaling pathways

The binding of RANKL to its receptor RANK in OC precursors

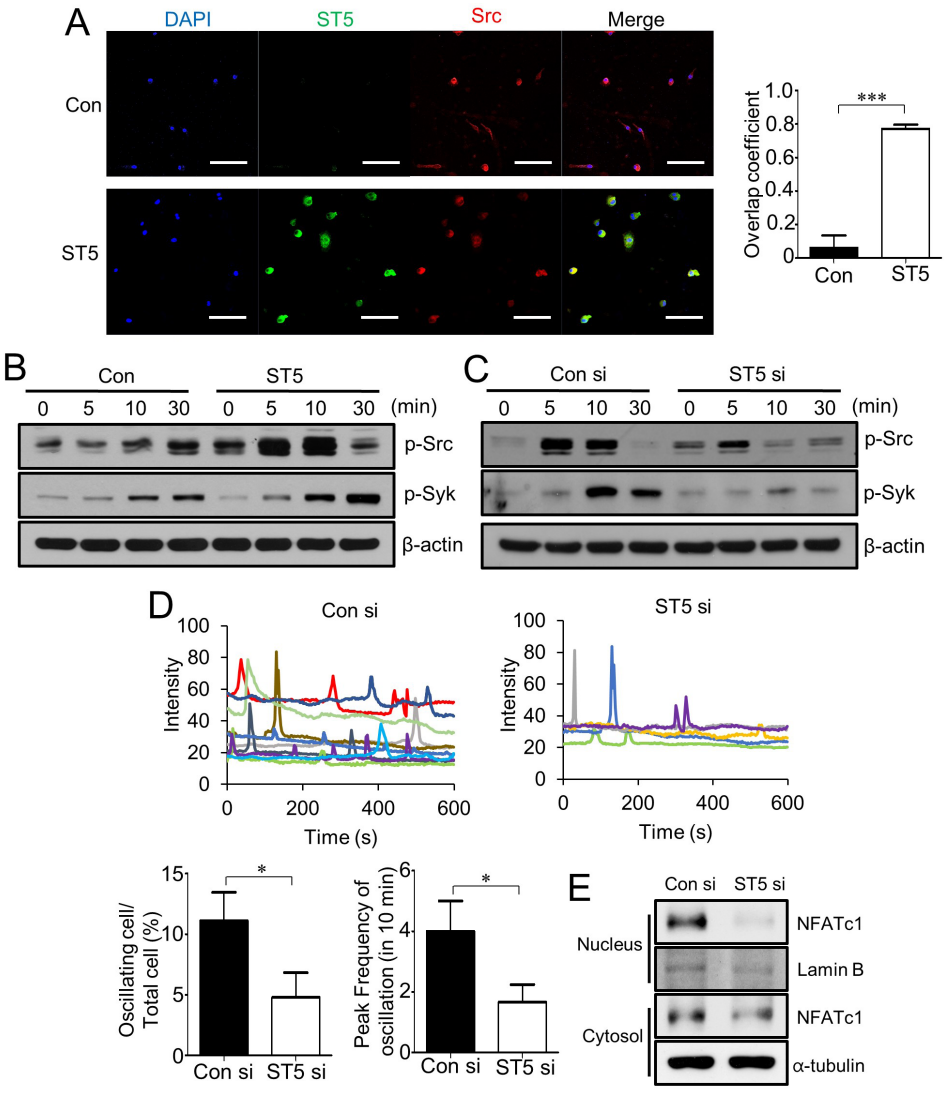

Fig. 5. ST5 mediates osteoclastogenesis via Src-Syk-calcium signaling pathways. (A) To quantify the overlapping region of cells between ST5 and Src, BMMs were overexpressed by a ST5 expression vector tagged with HA and treated with M-CSF (30 ng/ml) and RANKL (150 ng/ml) for 2 days. Cells were subjected to immunofluorescence. The fluorescence intensity of Cy3 (anti-Src)- and FITC (anti-HA)-labeled cells was analyzed by confocal microscope. The images of four microscopic fields per sample were randomly captured. The overlapped coefficient values of Cy3 and FITC among 32 cells per sample were determined by Zen 2009 software (Carl Zeiss). The experiments were performed in triplicate wells per group. Scale bars = 100 μm. (B and C) Transfected BMMs were starved for 5 h and treated with RANKL (500 ng/ml) for the indicated times. Protein levels of phospho-forms and total forms of Src and Syk. (D and E) ST5-deficient cells were incubated for 2 days with M-CSF (30 ng/ml) and RANKL (150 ng/ml). (D) To measure Ca^{2+} oscillations in individual cells, cells were loaded with Fura-2/AM for 30 min and monitored by a confocal laser. Each colored line represents calcium oscillation in a cell. (E) BMMs transfected with siRNA were stimulated with M-CSF (30 ng/ml) and RANKL (150 ng/ml) for 3 days. The levels of nuclear and cytoplasmic NFATc1 proteins were assessed by Western blotting. $*P < 0.05$, $***P < 0.001$ (by *t*-test).

leads to activation of MAPK and NFκB signaling pathways (Park et al., 2017; Takayanagi, 2005). To investigate the signaling pathways mediated by ST5, we next evaluated the effects of ST5 on RANKL-induced MAPK and NFκB signaling pathways. BMMs were stimulated with a high concentration of RANKL after serum starvation, and phosphorylation of p38, ERK, JNK, and IκB was analyzed by Western blotting. In the control and ST5 siRNA, phosphorylation of p38, ERK, and JNK MAPKs was detected at similar levels (Fig. 4A). Moreover, phosphorylation and degradation of IκB representing activity of canonical NFκB signaling were similar between the two groups (Fig. 4A). Consistently, there was no significant difference in phosphorylation of MAPKs and IκB by ST5 overexpression compared with that of the control (Fig. 4B).

ST5 positively regulates osteoclastogenesis via Src/Syk/calcium signaling pathways

The N-terminal domain of ST5 physically binds SH3, a domain present in the Src protein (Majidi et al., 1998). Moreover, Src is a key factor of osteoclastogenesis (Destaing et al., 2008; Schwartzberg et al., 1997). Therefore, to gain evidence of the molecular targets affected by ST5, we examined whether there was any association between ST5 and Src in OC precursors. To accomplish this, BMMs overexpressing the ST5 gene were cultured with RANKL for 2 days and then subjected to immunocytochemistry to detect ST5 and Src co-localization. We observed that ectopic ST5 protein was localized mainly in the cytoplasm, and the level of Src protein is similar in the control and ST5 groups (Fig. 5A). Intriguingly, we found that the ST5-overexpressed group has more double-positive cells labeled both green (anti-ST5) and red (anti-Src) than the control group (Fig. 5A). These results indicate an association between ST5 and Src, with ST5 possibly contributing to induction of OC differentiation via Src.

RANKL phosphorylates and activates Src, and activated Src kinase forms a complex with Syk, leading to phosphorylation and thereby activation of Syk. Syk, in turn, triggers calcium oscillation via PLCγ, which is required for activation and induction of NFATc1 in OCs (Kim et al., 2012; Oh et al., 2007; Tomomura et al., 2012; Yoon et al., 2009). Therefore, we hypothesized that ST5 mediates NFATc1 through Src-Syk-calcium signaling pathways. We first examined the role of ST5 in activation of Src and Syk. Consistent with previous reports (Arias-Salgado et al., 2003; Tomomura et al., 2012), BMMs gradually increased the phosphorylation levels of Src and Syk in response to RANKL (Fig. 5B). When cells overexpressed the ST5 gene, the phosphorylation levels of Src and Syk were strongly elevated compared to the control (Fig. 5B). Conversely, we observed a decrease in phosphorylation of Src and Syk in ST5 knockdown cells (Fig. 5C). Next, we examined whether ST5 regulates intracellular calcium signaling by RANKL. BMMs transfected with ST5 siRNA were cultured with RANKL for 2 days, and then intracellular calcium concentration was measured. As shown in Figure 5D, the amplitude and frequency of calcium peaks in the oscillation response were significantly lower in ST5 knockdown cells.

Calcium signaling mediates translocation of the NFATc1 transcription factor into the nucleus, where it binds to the promoter of osteoclastogenesis-related genes and regulates these genes (Kim and Kim, 2014; Koga et al., 2004). To examine the effect of decreased calcium signaling by ST5 knockdown on translocation of NFATc1 to the nucleus, we assessed the expression level of NFATc1 in the nuclear and cytoplasmic fractions. As shown in Figure 5E, ST5-deficient cells had a lower level of nuclear NFATc1 than control cells. Based on these analyses, it is reasonable to conclude that enhancement of Src-Syk-calcium signaling pathways by ST5 is crucial for increasing the probability of NFATc1 activity in the nucleus to induce OC marker genes.

DISCUSSION

In this report, we demonstrate that ST5 is a positive regulator of osteoclastogenesis. In microarray data using primary OC precursors, we found that the expression of ST5 is significantly increased during RANKL-osteoclastogenesis. In loss- and gain-of-gene approaches, we found that ST5 increases OC differentiation and expression of NFATc1. Moreover, our results indicate that ST5 activates Src/Syk/calcium signaling and enhances translocation of NFATc1 into the nucleus.

Binding of RANKL to RANK induces TRAF6-involved MAPK (ERK, JNK, and p38) and NFκB signaling, and cooperation between RANKL and the costimulatory signals of RANK synergistically activates Src/Syk and calcium signaling in OC precursors and OCs (Koga et al., 2004; Takayanagi, 2005; Tomomura et al., 2012). Despite involvement of ST5 in activation of MAPK/ERK2 signaling (Majidi et al., 1998; 2000), our results reveal that reduced or enhanced expression of ST5 does not affect phosphorylation of MAPKs including ERK (Fig. 4). Instead, we found that ST5 has relevance to Src. Previously, ST5 p126 was reported to bind the SH3 domain of Src in Cos-7 cells (Majidi et al., 1998). In our results, ST5 enhanced activation of Src and the Src substrate Syk by RANKL in OC precursors. In addition, ST5 knockdown decreases calcium oscillation (Fig. 5). Our results indicate that Src/Syk/calcium signaling participates in regulation of osteoclastogenesis by ST5.

ST5 p126 has a proline-rich domain with a PXXP core motif (Majidi et al., 1998). The PXXP sequence recognizes and binds SH3 and contributes to enhancing the kinase activity of Src and Abl (Alexandropoulos and Baltimore, 1996; Kurochkina and Guha, 2013; Ren et al., 1993; Yadav and Miller, 2007). Interestingly, the region between the two proline-rich regions of ST5 also binds SH3 of Src, independent of the PXXP motif (Majidi et al., 1998). We found that ST5 co-localizes with Src in differentiating OCs and enhances activation of Src signaling (Fig. 5). Src has SH2, SH3, and kinase catalytic domains and is repressed by intramolecular interactions between SH2 and SH3 domains and the kinase domain. SH3 ligand such as the proline-rich PXXP motif causes release of this interaction and increases the kinase activity of Src (Alexandropoulos and Baltimore, 1996; Yadav and Miller, 2007). We do not currently know whether ST5 activates Src by binding to the SH3 domain. Another possibility is a mechanism that involves Grb2. A previous report showed that the proline-rich region of ST5 p126 binds the SH3 domain of c-Abl and Grb2 (Majidi et al., 1998). In addition, Grb2 has been shown to activate Src by physically linking the non-receptor

isoform of protein-tyrosine phosphatase ε (cyt-PTPe) (Levy-Apter et al., 2014). Therefore, ST5 may indirectly activate Src via Grb2/cyt-PTPe or directly activate Src by binding to the SH3 domain. Although we did not precisely determine the interaction sites of ST5 and Src, we for the first time reveal a unique association between ST5 and Src in OCs.

In addition to binding with Src, ST5 is known to bind with Abl and Grb2 (Majidi et al., 1998). However, the results of experiments involving knockdown or overexpression of the ST5 gene seem to indicate that ST5 has little relevance to Abl in the process of OC differentiation. In a report on the effect of Abl in bone metabolism, $Abl^{-/-}$ mice show osteoporosis due to the effect of osteoblast maturation. This report shows that the OCs of $Abl^{-/-}$ mice function normally *in vitro* and in a histomorphometry study. However, differentiation of calvarial osteoblasts of $Abl^{-/-}$ mice is defective (Li et al., 2000). Therefore, a previous report revealing the non-function of Abl in OCs and our results showing the role of ST5 in OCs indicate little possibility of an Abl influence on OC regulation by ST5. Moreover, we propose that there is a relationship between ST5 and Grb2 in OCs. In a previous study, Grb2 was reported to be a positive regulator of OC. Knockdown of Grb2 results in a phenotype of disruptive OC formation and cytoskeletal organization and decreased activation of Src and Src-mediated downstream signaling (Levy-Apter et al., 2014). Similarly, our results reveal that ST5 enhances OC differentiation and activates the Src-mediated signaling pathway. In addition, we also observed that ST5 knockdown decreases the F-actin ring of mature OC (data not shown). Thus, we suggest that ST5 enhances OC formation by directly or indirectly regulating activation of Src-mediated signaling.

In our study, we found that ST5 regulates NFATc1 expression without the effect of c-Fos expression and also did not affect activation of NFκB, a known transcription factor of NFATc1 (Kim and Kim, 2014) (Figs. 2D, 3C, and 4). Therefore, because our results reveal that ST5 controls the activation of Src/Syk/calcium signaling (Figs. 5A-5D), we hypothesized that ST5 enhances NFATc1 expression via Src/Syk/calcium signaling. It is reported that Src and Syk play crucial roles in osteoclastogenesis (Destaing et al., 2008; Schwartzberg et al., 1997; Yamasaki et al., 2014). RANKL causes Src-mediated Syk phosphorylation and activation, and Syk activation by Src evokes calcium oscillation via activation of PLCγ, which leads to binding of IP3 to IP3 receptors located on the ER in OCs (Koga et al., 2004; Tomomura et al., 2012; Yamasaki et al., 2014). Then, calcium oscillation promotes translocation of NFATc1, a master transcription factor of OC differentiation, to the nucleus, leading to promotion of several OC-related genes such as TRAP, cathepsin K, and calcitonin receptor (Kim and Kim, 2014). Our results show that the level of NFATc1 in the nuclear fraction is decreased by ST5 knockdown in differentiating OCs (Fig. 5E). Moreover, NFATc1 is known to activate a positive amplification loop to maintain sufficient expression (Asagiri et al., 2005). Hence, these results reveal that ST5 enhances NFATc1 induction by activation of Src/Syk/calcium signaling and promotes NFATc1 transcription by autoamplification.

In conclusion, we demonstrate that enhanced expression of ST5 by RANKL is important in OC differentiation. In addition, we illustrate that increased ST5 expression by RANKL enhances Src/Syk/calcium signaling and then induces NFATc1 activation by promoting nuclear translocation and autoamplifying an NFATc1-dependent transcriptional program. Subsequently, activated NFATc1 increases the levels of OC marker genes such as TRAP and other factors, leading to enhanced osteoclastogenesis (Fig. 6). Therefore, we propose that decreasing the level of ST5 expression is a useful strategy for treating osteoporotic diseases.

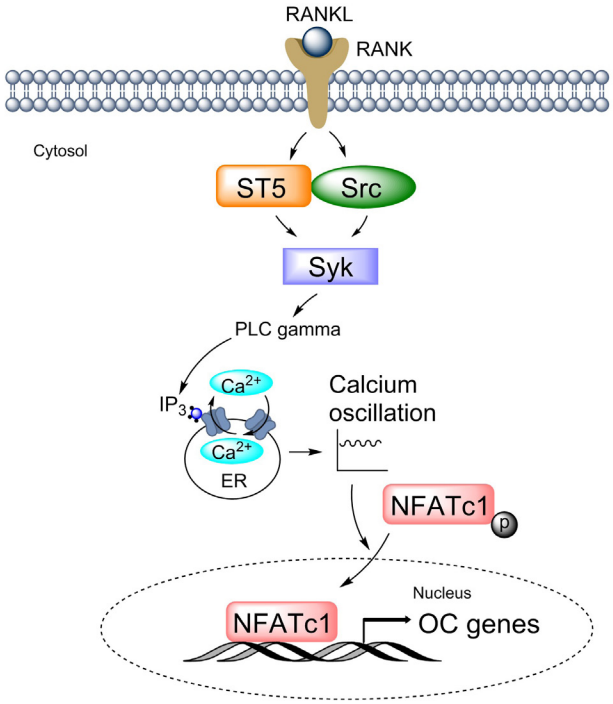

Fig. 6. The role of ST5 in osteoclast differentiation. ST5 induction by RANKL stimulates activation of Src/Syk signaling. Subsequently, transduction of IP3 by PLC-γ signaling induces calcium oscillation by binding the IP3 receptor on the ER, which increases NFATc1 activation, thereby enhancing osteoclast differentiation by promoting transcription of osteoclast marker genes.

Disclosure

The authors have no potential conflicts of interest to disclose.

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