The role of Pellino-1 as a regulator of NFATc1 signaling during osteoclast differentiation

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This certifies that the Master's Thesis of
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정수진 올림

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ABSTRACT

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(Directed by Professor Jin Woo Lee)

Bone homeostasis requires an adequate balance of bone formation by osteoblasts and bone resorption by osteoclasts. Failure to this coupling process, it originates diseases such as osteoporosis. Many studies reported that osteoclast differentiation is associated with various signal pathway. Pellino-1 is a E3 ligase protein which is well known as a regulator in cell signal pathway through ubiquitination of target proteins. In this study, I investigated the role and mechanism of Pellino-1 during osteoclastogenesis. Here I demonstrated that the loss of Pellino-1 in osteoclasts lead to excessive osteoclastogenesis through

inactivation of GSK-3β.

in vivo study showed that the loss of Pellino-1 gene induced osteoclast differentiation and reduced bone mineral density in Pellino-1 knockout mice. Knockout of Pellino-1 in osteoclasts also increased the secretion of the bone resorption marker CTX-1. To find out the mechanism of Pellino-1 during osteoclast differentiation, I performed knockdown study of Pellino-1, then found that NFATc1 promoter activity was increased and inactive form of GSK-3β was upregulated. Also, by ubiquitination assay, Pellino-1 turned out to be inducing ubiquitination of GSK-3β. These results proposed that Pellino-1 regulates the activity of GSK-3β in osteoclast differentiation. In conclusion, I have described a novel mechanism of Pellino-1 as a negative regulator in osteoclast differentiation. Furthermore, this study would be offer a new therapeutic target for dysregulated bone remodeling including osteoporosis and bone fracture.

Key words: Pellino-1, Bone homeostasis, Osteoclastogenesis, Osteoporosis, NFATc1, GSK-3β

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I. INTRODUCTION

Bone is a dynamic tissue which supports of soft tissues, levers for muscle action, protects of the central nervous system, releases of calcium and other ions for the maintenance of a constant ionic environment in the extracellular fluid. To accomplish its functions, bone undergoes continuous destruction carried out by osteoclasts, and formation by osteoblasts[1]. This process is called bone remodeling and maintain bone homeostasis by balance between osteoclasts and osteoblasts. But this bone homeostasis can be broken by various causes such as aging, changes

in the hormone, etc[2]. The imbalance in bone remodeling caused by abnormal osteoclast differentiation may result in skeletal diseases, such as osteoporosis and osteopetrosis[3].

Osteoporosis is a common bone metabolic disease, characterized by systemic bone loss, disordered bone microstructure, and increased fragility[4]. An imbalance in bone homeostasis is responsible for osteoporosis, but mainly for excessive osteoclast activity. Bone absorption and formation are closely related, so if bone absorption is inhibited, bone formation is suppressed[5]. So, it is to be in the limelight that develop a treatment for osteoporosis that works even if taken for a long time and does not adversely affect each other's coupling process.

Osteoclasts are multinucleated cells that differentiate from the monocytes / macrophages of hematopoietic lineage cells by two important cytokines, macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB (NF-κB) ligand (RANKL)[2]. M-CSF induces proliferation and survival of osteoclast precursors, and RANKL forms TNF receptor associated factor 6 (TRAF6) complex to activate NF-κB and mitogen-activated protein kinase (MAPK) so that the osteoclast precursors can be differentiated into mature osteoclasts that can act bone-absorbing. In this process, forming TRAF2, 5 and 6 complex is an essential process for Nuclear factor of activated T-cells (NFATc1) and NF-κB signaling activation[6,7]. TRAF6 is the major protein that regulates RANK-associated signaling during osteoclast differentiation and activity[8,9]. Furthermore, TRAF6 knockout mice showed osteopetrosis due to a defect of

osteoclast differentiation and occurred osteopetrosis[10]. The E3 ligase, Pellino-1 is noted for forming this TRAF6 complex by ubiquitinates Interleukin-1 receptor-associated kinase 1 (IRAK1) and TRAF6 under the inflammatory environment as Toll-like receptor (TLR)[11,12]. Therefore, I could conclude that Pellino-1 is essential to the formation of the TRAF6 complex. Furthermore, bone homeostasis is influenced by inflammatory environments such as NF-κB, so it could be thought that Pellino-1 would have an effect, but the research related to Pellino-1 and bone has been insufficient.

Pellino-1 protein is an enzyme including Wing, FHA domain and RING-like domain and is known for ubiquitination of IRAK1 and TANK-binding kinase 1 (TBK1)[13,14]. Ubiquitination is one of the post-translational modification that modulates the activity of various proteins. The ubiquitin conjugation cascade is processed with the help of three enzymatic components, ubiquitin activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3)[15,16]. Pellino-1 is mainly well known to facilitate signaling complex formation in the TLR and interleukin-1 receptor (IL-1R) signaling pathways by promoting the ubiquitination of the kinase receptor-interacting protein 1 (RIP1), IRAK 1 and the downstream activation of NF-κB[14,17]. Pro-inflammatory cytokines induce osteoclast differentiation and inhibit osteoblast maturation[18-20]. Although it is expected that Pellino-1 will play a crucial role in bone homeostasis because the activity of osteoblast and osteoclast is controlled by these inflammatory reaction, the research on this have not been investigated.

Glycogen synthase kinase 3 beta (GSK-3β) is a serine/threonine kinase originally identified as a regulator of glycogen deposition[21]. The activation of Wnt/β`–catenin signals in osteoblast lineage cells such as osteoblasts and osteocytes induces the expression of osteoprotegerin and then inhibits osteoclast formation[22]. GSK-3β is inactivated by RANKL stimulation via serine phosphorylation during osteoclastogenesis, and that activates expression and nuclear localization of NFATc1[21].

In this study, I expected that the interaction between Pellino-1 and GSK-3β is crucial in regulation of osteoclastogenesis. I revealed that Pellino-1 is negative regulator of osteoclast differentiation via ubiquitination of GSK-3β. Thereby, this study will suggest the role of Pellino-1 during osteoclastogenesis and introduce a new mechanism to alleviate disproportionate osteoclast differentiation.

II. MATERIALS AND METHODS

1. Mouse generation

Mice lacking *Pellino-1* in osteoclasts using *Cathepsin K* (*Ctsk*)-Cre mice were generated by intercrossing *Pellino-1*$^{flox/+}$, *Cathepsin K*-Cre breeders with *Pellino-1*$^{fl/fl}$ mice (referred to herein as *Pellino-1*^{Ctsk}) to investigate *in vivo* function of Pellino-1 in osteoclasts. *Pellino-1*$^{flox/+}$ mice were from M. Lee (Yonsei University College of Medicine, Seoul, South Korea). *Cathepsin K*-cre mice were a gift from J. Shim (Weill Cornell Medical College, New York, USA)[23]. All mice were on a C57BL/6 background. All experimental procedures in this study were approved by the Institutional Animal Care and Use Committee of Yonsei University Health System, Korea. The protocol was approved by the Committee on the Ethics of Animal Experiments of Yonsei University College of Medicine (Permit Number: 2019-0255). All experimental procedures in this study were approved by the Institutional Animal Care and Use Committee of Yonsei University Health System.

2. Isolation of mBMMS

Mouse bone marrow cells from 6-week-old male *Pellino-1*$^{fl/fl}$ and *Pellino-1*^{Ctsk} mice were isolated from femur and tibia by flushing the bone marrow with 27-gauge needles, and filtered with a 70-μm cell strainer (Falcon, Tewksbury, MA, USA). Cells were centrifuged at 1,500 rpm for 10 minutes and re-suspended in α-MEM medium containing 10% FBS, 1% penicillin 50 units/ml. Cells were

seeded in 75T flask culture dish. After 72 hours, the supernatant was collected and seeded into a new 10-cm^2 culture dish. The supernatant was re-collected and centrifuged at 1,500 rpm for 10 minutes. Mouse bone marrow-derived monocytes (BMMs) were cultured in α-minimum essential media (α-MEM; Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco) with 1% antibiotic-antimycotic solution (Gibco). Then incubated mBMMs in 5 % CO_2 at 37℃ atmosphere.

3. Micro-CT

For μCT analysis, femoral samples were fixed in 70% ethanol for 24 hours at room temperature. The fixed samples were analyzed using high-resolution μCT (Skyscan-1173, Bruker-CT, Kartuizersweg, Belgium). μCT image reconstruction and analysis was performed using reconstruction software Nrecon (v1.7.0.4, Bruker-CT) and obtained the image of 2240x2240 Pixel. The cross-section image was arranged using the DataViewer (v1.5.1.2, Bruker-CT). As for the analysis program, Ct Analyzer (v1.14.4.1, Bruker-CT) was used to separate trabecular bone and bone marrow from the images respectively. The software used in the measurement is SkyScan1173 control software (v1.6, Bruker-CT), and the conditions of the imaging were voltage of 90 kVp and current 88 μA and a 1.0 mm-thick aluminum filter. The pixel size was 7.83 μm, exposure time was 500 ms, the rotation step was 0.3°, and full rotation occurred over 180° for obtaining a total of 800 high resolution images. The measured 3D bone parameters of 6-week-old

mice included the following: trabecular bone per tissue volume (BV/TV); trabecular number (Tb. N); trabecular thickness (Tb. Th); trabecular separation (Tb. Sp); bone mineral density (BMD).

4. Osteoclast differentiation

mBMMs were detached using Detachin™ (Genlantis, San Diego, CA, USA) and 1×10^5 cells were seeded on 12-well plate for osteoclastogenesis. mBMMs were cultured in growth media containing 10 ng/ml M-CSF and 10 ng/ml RANKL to induce osteoclastogenesis (R&D Systems, Minneapolis, MN, USA). The induction medium was changed every other day.

5. TRAP activity and staining

osteoclast culture supernatant 100 μl was incubated with substrate mix that containing acetate solution (Sigma-Aldrich, St.Louis, MO, USA), 1M sodium tartate and phosphatase substrate (Sigma-Aldrich) for 1 hour at 37℃. The reaction was stopped by adding 3N HCl. TRAP activity was measured at absorbance wavelength of 405 nm. TRAP staining was performed using Acid Phosphatase, Leukocyte kit (Sigma-Aldrich) following the manufacturer's instructions. Cells were fixed with fixative solution and stained with staining solution for 1 hour at 37℃.

6. Whole-mount skeletal staining

New born mice were sacrificed, skined off, removed organs except brain. Fix the mice in 95% EtOH overnight at room temperature. Next day, Place the samples in acetone overnight at room temperature. Stain for cartilage and bone by Alcian blue solution (Sigma-Aldrich) and Alizarin red S solution (Signa-Aldrich), relatively for one day at 37℃ incubator and next day, replace that solution at room temperature. Destain mice by initially washing them in 95% EtOH for 2 hours at room temperature. And to pre-clear the tissue, add 1% KOH solution. Transfer sample in 80% glycerol/0.02% sodium azide for long-term storage.

7. Immunohistochemical analysis

The femoral samples from each group were fixed in 10% formalin solution for 5-7 days at room temperature. Samples were decalcified in 0.5M EDTA (pH 7.4) solution for 2 weeks at room temperature. Decalcified femurs were embedded in paraffin blocks. The paraffin sections were dehydrated by passage through an ethanol series, cleared twice in xylene, and embedded in paraffin, after which 5 mm sections were cut using a rotary microtome. Decalcified femoral sections were stained with Haemotoxylin and Eosin, Masson’s Trichrome, Safranin O and Fast Green. For immunohistochemistry, antigen retrieval was performed using citrate buffer (pH 6.0) for the deparaffinized section. Sections were blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature. And then, sections were incubated with TRAP antibody. DAB Substrate Kit (ab64238,

Abcam, Cambridge, United Kingdom) was used for detection TRAP positive cells on tissue sections.

8. Gene transfection and luciferase reporter assay

Recombinant plasmids were transfected into HEK293T cells using X-tremeGENE 9 DNA Transfection Reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol. Then, the medium was replaced with 2 ml DMEM-HG with 10% FBS and 1% antibiotic antimycotic solution. After 24 hours, the cells were lysed with 200 μl of 1× passive lysis buffer (Promega, Madison, WI, USA) per tube, and cell debris was removed. Luciferase activity was measured by the dual-luciferase reporter assay kit (Promega). Relative luciferase activity was normalized by renilla activities to adjust transfection efficiency. The assays were performed at least three times.

9. Western blotting

mBMMs pellets were suspended and lysed in Pro-Prep protein extraction solution (Intron, Seongnam-si, Korea). The protein concentrations were measured using BCA protein assay kit (Intron), and 30 μg of protein was analyzed by loading samples onto the 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Then proteins were transferred onto the polyvinylidene difluoride (PVDF) membranes (Hybond Escondido, CA, USA). Once all the proteins were transferred onto the membrane, the membrane was

blocked with 5% skim milk (BD Biosciences, San Jose, CA, USA) for 1 hour at room temperature. After blocking period, the membrane was incubated for 4 hours at room temperature or over-night at 4℃ with primary antibody.

10. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was obtained using AccuPrep® Universal RNA Extraction Kit (Bioneer, Korea) followed by the manufacturer's instruction. The total RNA was reversely transcribed using Omniscript kit (Qiagen, Hilden, Germany). qRT-PCR was performed to estimate the changes of mRNA expression between groups. The primer sets used were as follow (Table 1).

11. Immunoprecipitation (IP)

Adequate number of HEK293T cells were seeded in 6 well culture plate with 70% confluence. Then, transfected HEK293T cells with pCMV-GFP, Pellino-1-GFP and GSK-3β using X-tremeGENE 9 DNA Transfection Reagent (Roche). After 4-5 hours of incubation, DMEM-HG mixed with 1 μM of MG-132 (proteasome inhibitor [Sigma]) were added for 24 hours, and then harvested to obtain at least 400 μg of protein. The harvested cells were lysed. The lysates were precipitated using protein A/G agarose beads (Santa Cruz Biotechnology, CA, USA) and antibodies GSK-3β (Santa Cruz Biotechnology) and A/G agarose beads with the lysates and antibodies were collected by centrifugation. The supernatants were removed from the beads. Then 2X SDS

sample buffer was added to obtain complexes from the beads by boiling the samples. The prepared samples were loaded into the 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Immunoblotting was done with antibodies as indicated.

12. Ubiquitination assay

HEK293T cells were transfected with ubiquitin, and GSK-3β, along with control vector, Pellino-1, and Pellino-1 small inhibitory RNAs (siRNAs; Bioneer, Daejeon, Korea). After 24 hours transfection, the cells were treated with 1 μM MG132 (proteasome inhibitor [Sigma]) for 24 hours then the cells were harvested. Cell lysates were immunoprecipitated and immunoblotted with Pellino-1 and GSK-3β antibodies.

13. ELISA

Blood was collected from 6-week-old male *Pellino-1*$^{fl/fl}$ and *Pellino-1*^{Ctsk} mice by cardiac puncture immediately after sacrifice. Serum was separated using serum separator tubes and serum levels of CTX (Immuno diagnostic systems, United Kingdom) were measured using each ELISA kits as per the manufacturer's instructions.

14. Statistical analysis

Statistical significance between groups were evaluated by using Student's t-tests or one-way ANOVA using GraphPad Prism6 software. Data were shown as the mean ± standard deviation. Values of $p < 0.05$ were considered statistically significant

Table 1. A list of primers used for qRT-PCR

Gene	Primer sequence (5' → 3')
Pellino-1	F : TGTGGGAACGTCTTCAGTCTGC
	R: CAGCAAGGTTGCACCACAAAGG
Pellino-2	F : GTGTGACAGGAACGAGCCATAC
	R: AGGACACCGTTGGTAGTGAGTC
Pellino-3	F : CTGCTGGCTTTGATGCCTCTAG
	R: GCAGAGTCCTCGGAGAAGCCA
Nfatc1	F : GGTAACTCTGTCTTTCTAACCTTAAGCTC
	R: GTGATGACCCCAGCATGCACCAGTCACAG
Cathepsin K	F : AGGCAGCTAAATGCAGAGGGTACA
	R: ATGCCGCAGGCGTTGTTCTTATTC
Dc-stamp	F : TCCTCCATGAACAAACAGTTCCAA
	R: AGACGTGGTTTAGGAATGCAGCTC
c-fos	F : GGAGAATCCGAAGGGAACGG
	R: GCAATCTCAGTCTGCAACGC
β-actin	F : CTTCTACAATGAGCTGCGTG
	R: TCATGAGGTAGTCTGTCAGG

III. RESULTS

1. Pellino-1 expression decreases during osteoclastogenesis in mBMMs

To assess if Pellino-1 has a role in the process of osteoclastogenesis, I isolated primary osteoclast pro-genitors from ICR mice and induced osteoclast differentiation *in vitro* by M-CSF and RANKL. During osteoclast differentiation (Fig. 1A, B), the expression of Pellino family decreased. And specifically, the expression of *Pellino-1* mRNA level exhibited a significant reduction (Fig. 1C). Protein level also showed down regulation of Pellino-1 during osteoclastogenesis (Fig. 1D). Taken together, these results demonstrate that Pellino-1 has a negative role during osteoclastogenesis in mBMMs.

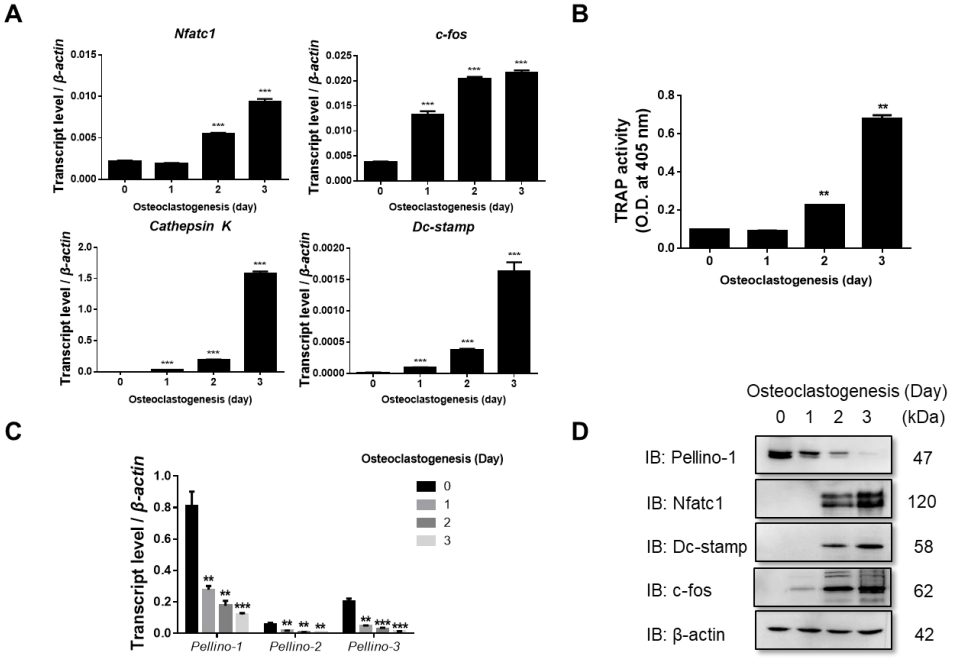

Figure 1. Pellino-1 expression is down-regulated during osteoclastogenesis. (A, B, C, D) mBMMs were cultured with 10 ng/ml of M-CSF and RANKL and harvested indicated day. Expression of osteoclast marker genes at different days were analyzed by qRT-PCR. (A) mRNA expression of Pellino family genes at different days were analyzed by qRT-PCR. (B) TRAP activity was measured at different days after RANKL treatment in mBMMs by colorimetric analysis. (C) mRNA expression of Pellino family genes at different days were analyzed by qRT-PCR. (D) Protein levels of Pellino-1 and osteoclast marker genes at different days were immunoblotted with the indicated antibodies. All error bars indicate S.D. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

2. Conditional deletion of *Pellino-1* in osteoclasts positive cells leads to low bone mass

I generate mice lacking *Pellino-1* in osteoclasts using *Cathepsin K* (*Ctsk*)-Cre mice to investigate *in vivo* function of Pellino-1 in osteoclasts (Fig. 2A). Mouse genotypes were confirmed by PCR (Fig. 2B) and the gene deletion was verified by western blotting (Fig. 2C). *Pellino-1*^{Ctsk} didn't affect survival and reproductive ability, the growth kinetics is comparable to *Pellino-1*$^{fl/fl}$ mice. From 5-week-old, the weight of *Pellino-1*^{Ctsk} was lower than *Pellino-1*$^{fl/fl}$, and it was confirmed that the size of *Pellino-1*^{Ctsk} was smaller than *Pellino-1*$^{fl/fl}$ at the 6-week-old. Also, the femur size was smaller in *Pellino-1*^{Ctsk} compared to *Pellino-1*$^{fl/fl}$ mice (Fig. 3A, B, C). However, there was no difference in newborn mice, which confirmed that the osteoclast activity regulation by Pellino-1 was carried out during postnatal development (Fig. 3D).

6-week-old *Pellino-1*^{Ctsk} mice femur exhibited a significant reduction of bone volume as compared to their controls. In trabecular bone, compared with the *Pellino-1*$^{fl/fl}$ mice, the *Pellino-1*^{Ctsk} mice were characterized by significantly decreased percentage bone volume (BV/TV), bone mineral density (BMD), trabecular thickness (Tb. Th), trabecular number (Tb. N), and trabecular separation (Tb. Sp) (Fig. 4A, B). The same in cortical bone, cortical Tb.Th, cortical Tb.N, cortical Tb. Sp in *Pellino-1*^{Ctsk} reduced than *Pellino-1*$^{fl/fl}$ (Fig. 5A, B). These finding suggest that *Pellino-1* deletion in osteoclasts induced excessive bone resorption.

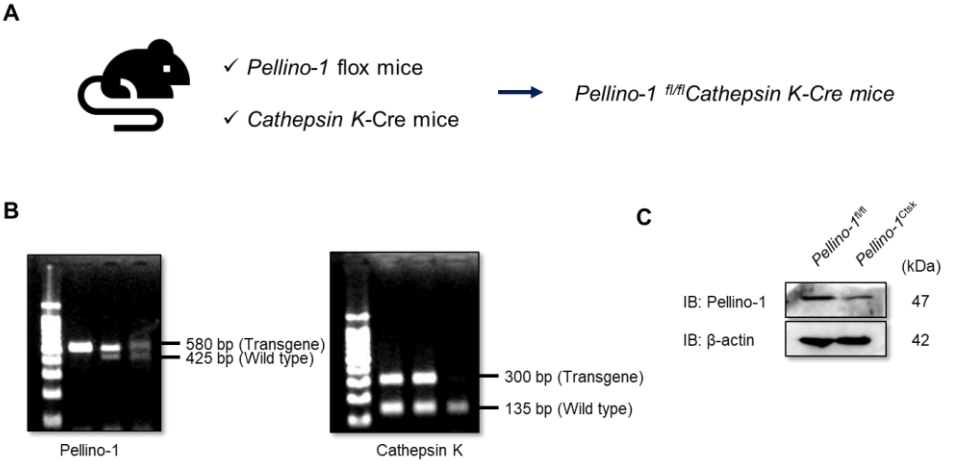

Figure 2. Generation of Pellino-1 conditional knockout mice. (A) *Pellino-1*$^{fl/+}$ mice and *Cathepsin K-Cre* mice are crossed to get *Pellino-1*^{Ctsk} mice. (B) PCR data showed Pellino-1 and Cathepsin K-Cre's transgene or wild type band. (C) Protein levels of Pellino-1 in *Pellino-1*$^{fl/fl}$ and *Pellino-1*^{Ctsk} mBMMs.

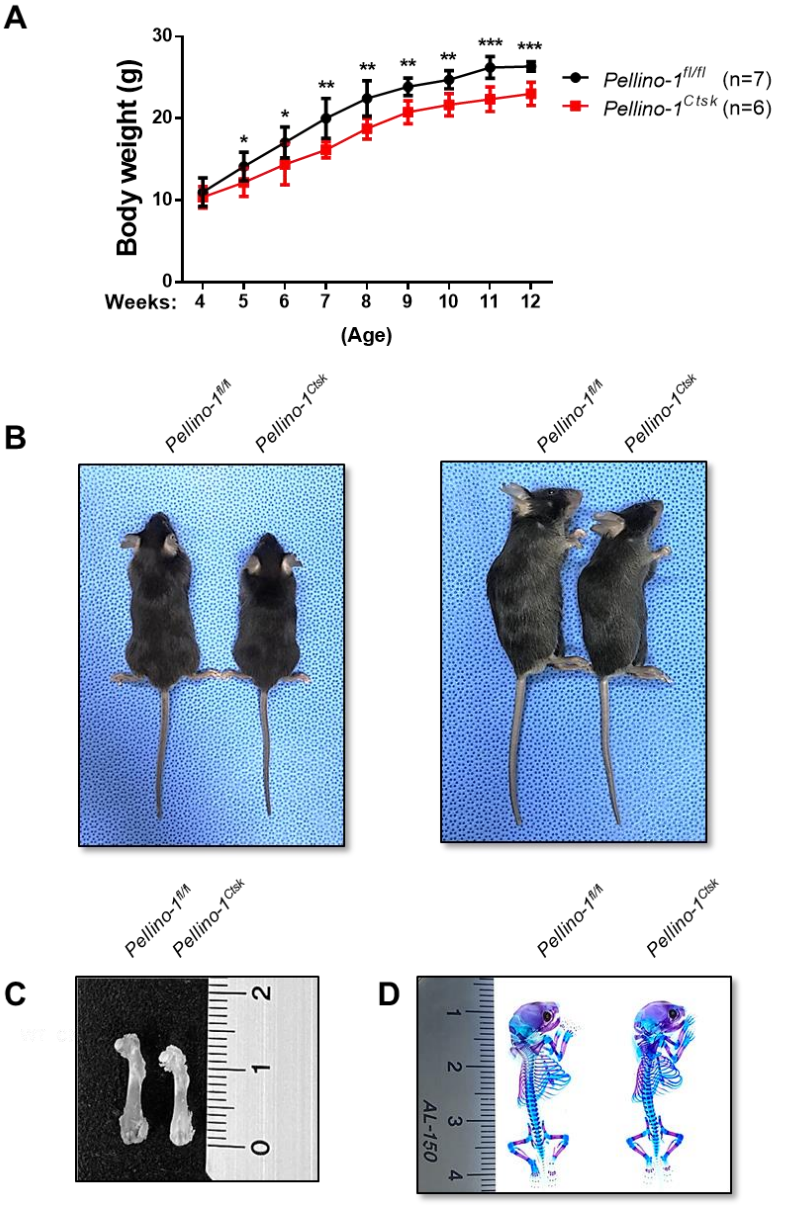

Figure 3. *Pellino-1*^{Ctks} mice are smaller than *Pellino-1*$^{fl/fl}$ mice. (A) Growth kinetics comparing *Pellino-1*$^{fl/fl}$ and *Pellino-1*^{Ctsk} mice aged between 4-week-old and 12-week-old. (B) Growth photo and (C) femur comparing 6-week-old *Pellino-1*$^{fl/fl}$ and *Pellino-1*^{Ctsk}. (D) Skeletal phenotype of new born *Pellino-1*$^{fl/fl}$ and wild *Pellino-1*^{Ctsk} mice. Whole-mount alizarin red S and alcian blue staining was performed. All error bars indicate S.D. (* $P < 0.05$, ** $P < 0.01$, * $P < 0.001$).

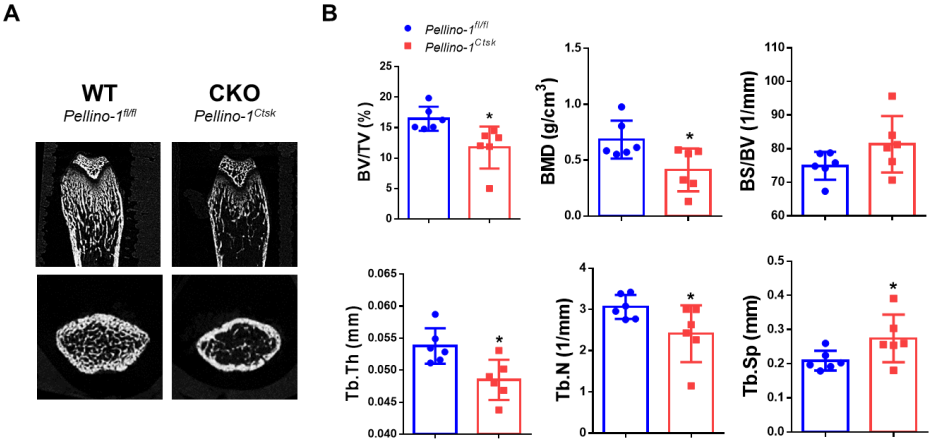

Figure 4. Conditional deletion of *Pellino-1* in osteoclasts leads to low bone mass in trabecular bone. (A) Representative image of micro-CT analysis of femur at 6-week-old male *Pellino-1*$^{fl/fl}$ and *Pellino-1*^{Ctsk} mice. (B) Quantitative micro-CT analysis of trabecular bone parameters at 6-week-old male *Pellino-1*$^{fl/fl}$ and *Pellino-1*^{Ctsk} mice including bone volume fraction ratio (BV/TV), Volumetric BMD of trabecular bone, bone surface ratio (BS/BV), trabecular thickness (Tb. Th), trabecular number (Tb. N), trabecular separation (Tb. Sp) are shown. (n = 6 for each group). All error bars indicate S.D. (* $P < 0.05$, n=6 per each group).

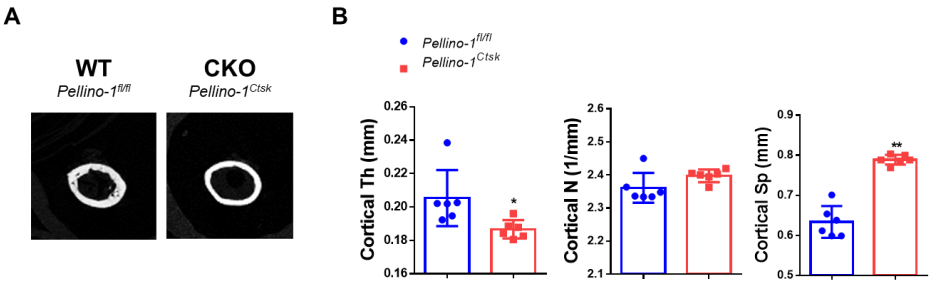

Figure 5. Conditional deletion of *Pellino-1* in osteoclasts shows decreased bone mass in cortical bone. (A) Representative image of micro-CT analysis of cortical bone at 6-week-old male *Pellino-1*$^{fl/fl}$ and *Pellino-1*^{Ctsk} mice. (B) Quantitative micro-CT analysis of cortical bone parameters at 6-week-old male *Pellino-1*$^{fl/fl}$ and *Pellino-1*^{Ctsk} mice including cortical bone trabecular thickness (Tb. Th), trabecular number (Tb. N), trabecular separation (Tb. Sp) are shown. (n = 6 for each group). All error bars indicate S.D. (* $P < 0.05$, ** $P < 0.01$, n=6 per each group).

3. Conditional deletion of *Pellino-1* in osteoclasts positive cells activates osteoclastogenesis

Confirming the multinuclear site, which is a characteristic of osteoclastogenesis through H&E staining, I found that there are more multinuclear sites in *Pellino-1*^{Ctsk} than *Pellino-1*$^{fl/fl}$ (Fig. 6A). Also, osteoclast activity was upregulated in *Pellino-1*^{Ctsk} through more TRAP-positive sites (Fig. 6B). A greater number of osteoclasts in *Pellino-1*^{Ctsk} (Fig. 6C). In addition, the serum level of c-terminal telopeptides of collage type I (CTX), a marker for bone resorption, was elevated in the *Pellino-1*^{Ctsk} group (Fig. 7).

To examine the effect of *Pellino-1* deletion in *ex vivo* osteoclast development, mBMMs isolated from *Pellino-1*$^{fl/fl}$ and *Pellino-1*^{Ctsk} mice were treated with M-CSF and RANKL. Osteoclast differentiation was significantly increased in *Pellino-1*^{Ctsk} BMMs compared to *Pellino-1*$^{fl/fl}$ BMMs, as evidenced by an increase in TRAP staining and activity. And *Pellino-1*^{Ctsk} osteoclasts were more enlarged than *Pellino-1*$^{fl/fl}$ osteoclasts (Fig. 8A, B). Consistently, gene expression of the key transcriptional factors of osteoclast differentiation including *Nfatc1, Ctsk, Dc-stamp, C-fos* was enhanced in *Pellino-1*^{Ctsk} BMMs after osteoclast induction for 4 days (Fig. 9A). Western blot analyses confirmed the augmented expression of Nfatc1, C-fos in *Pellino-1*^{Ctsk} BMMs after osteoclastic induction for 4 days (Fig. 9B).

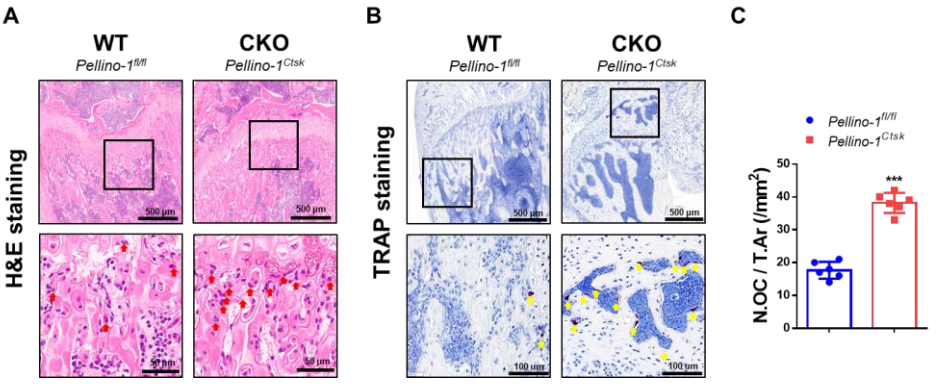

Figure 6. Knockout of *Pellino-1* activates osteoclastogenesis. (A, B) Representative histological analysis of paraffin sections of femur from *Pellino-1*$^{fl/fl}$ and *Pellino-1*^{Ctsk} at 6 weeks old. (A) Hematoxylin and eosin staining. (B) TRAP staining. Red arrows indicate multi nuclear sites. Yellow arrows indicate TRAP positive area. (C) Quantification of osteoclast number/bone surface (N.Oc/BS) and osteoclast surface/bone surface (Oc.S/BS). All error bars indicate S.D. (*** $P < 0.001$).

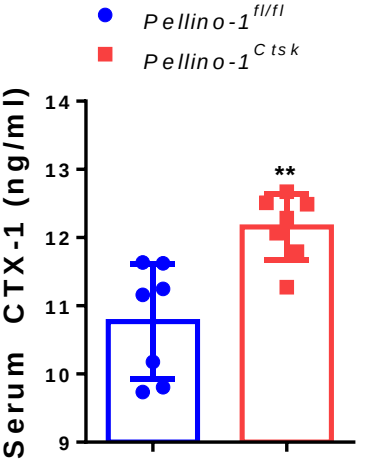

Figure 7. Knockout of *Pellino-1* elevates bone resorption activity. The levels of serum bone resorption index, CTX-1, were detected by ELISA. All error bars indicate S.D. (** $P < 0.01$, n=7 per each group).

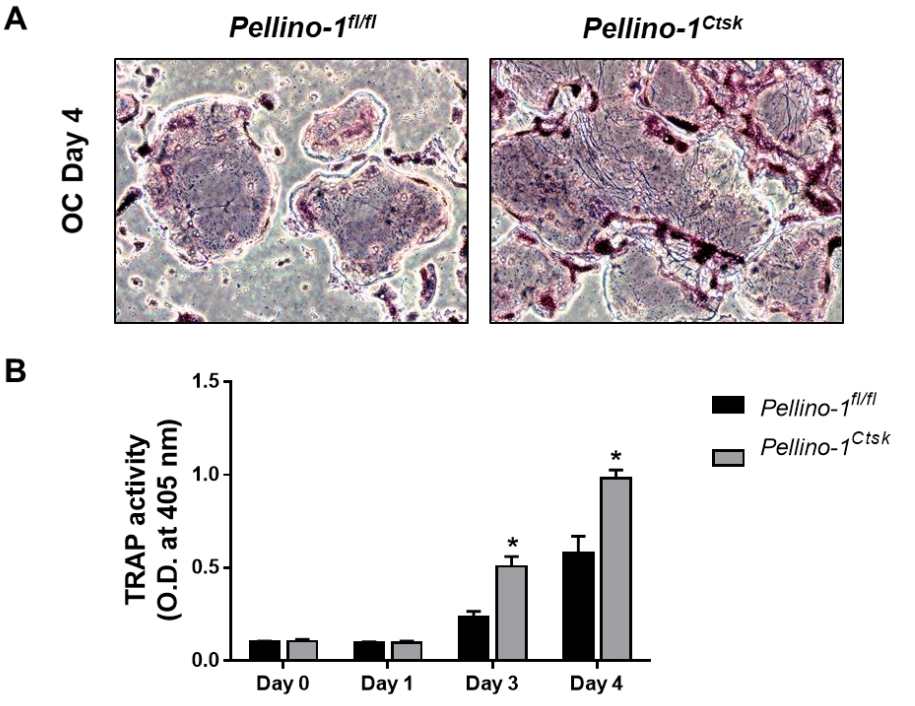

Figure 8. Knockout of *Pellino-1* increases osteoclastogenesis in *ex vivo*. (A, B) *Pellino-1*$^{fl/fl}$ and *Pellino-1*^{Ctsk} BMMs were cultured with 10 ng/ml of M-CSF and RANKL and multi-nucleated cells were stained with (A) TRAP and measured with (B) TRAP activities at day 4 of culture. All error bars indicate S.D. (* $P < 0.05$).

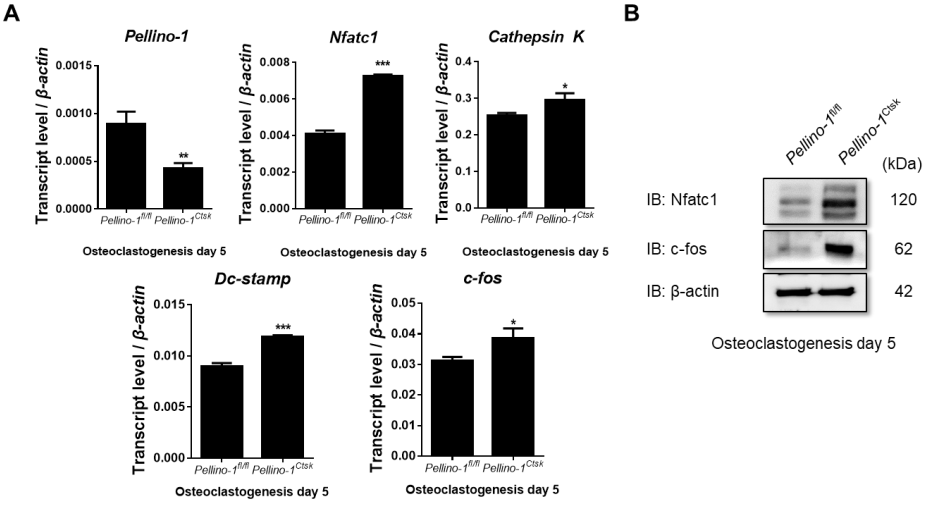

Figure 9. Knockout of *Pellino-1* upregulates osteoclast marker genes during osteoclastogenesis. (A, B) mBMMs isolated from *Pellino-1*$^{fl/fl}$ and *Pellino-1*^{Ctsk} were cultured with 10 ng/ml of M-CSF and RANKL and harvested at 4th day after osteoclast induction. (A) mRNA Expression of osteoclast marker genes were analyzed by qRT-PCR. (B) Protein levels of osteoclast marker genes were immunoblotted with the indicated antibodies. All error bars indicate S.D. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n=6 per each group).

4. Knockdown of Pellino-1 increases NFATc1 activity

The data shown above is enough to say that Pellino-1 regulation is necessary to activate osteoclast differentiation. Herein, I would like to find out the mechanism how Pellino-1 controls osteoclastogenesis. First of all, I checked the two most crucial transcription factor for osteoclast differentiation, NF-κB and NFATc1's transcriptional activity. In the case of NF-κB, it decreased during Pellino-1 knockdown. But in contrast, NFATc1 transcriptional activity increased (Fig. 10A, B). This pattern also detected in protein level after Pellino-1 knockdown (Fig. 10C).

GSK-3β facilitates NF-κB and inhibits NFATc1 activity[24]. GSK-3β promotes NF-κB by phosphorylating NF-κB essential modulator (NEMO), and impedes NFATc1 by suppressing the nuclear translocation of endogenous NFATc1 components[25]. Meanwhile, NFATc1 is also deeply related to GSK-3β during the osteoclastogenesis. After RANKL treatment, GSK-3β was highly phosphorylated at Ser-9, causing inactivation of GSK-3β so that NFATc1 transfer well into nuclear[26]. Accordingly, I could hypothesize that GSK-3β would not be activated as the ubiquitination due to reduced Pellino-1 expression level under osteoclastogenesis, resulting in NFATc1 activation and accelerates osteoclast differentiation. Ubiquitination can regulate activity of kinase protein[27,28]. To confirm the Pellino-1 function as E3 ubiquitin ligase, the ubiquitination assay was performed. Knockdown of Pellino-1 showed significantly inhibited ubiquitination of GSK-3β (Fig. 11A). Conversely, overexpression of Pellino-1 increased

ubiquitination of GSK-3β (Fig. 11B). These results revealed that Pellino-1 is a crucial E3 ligase that regulates the activity of GSK-3β.

As Pellino-1 regulates the activity of GSK-3β through ubiquitination, I wanted to see if the phosphorylation of GSK-3β also changed accordingly. Knockdown of Pellino-1 using siRNA lead to upregulation of Ser-9 phosphorylated GSK-3β. And NFATc1 protein level was upregulated in compared with control group (Fig. 12A). Decreased phosphorylation of NFATc1 supported that NFATc1 became activate form (Fig. 12A). The same results are shown in mBMM during osteoclastogenesis, suggesting that Pellino-1 knockout positively regulates osteoclast differentiation via down-regulated GSK-3β activity. (Fig. 12B).

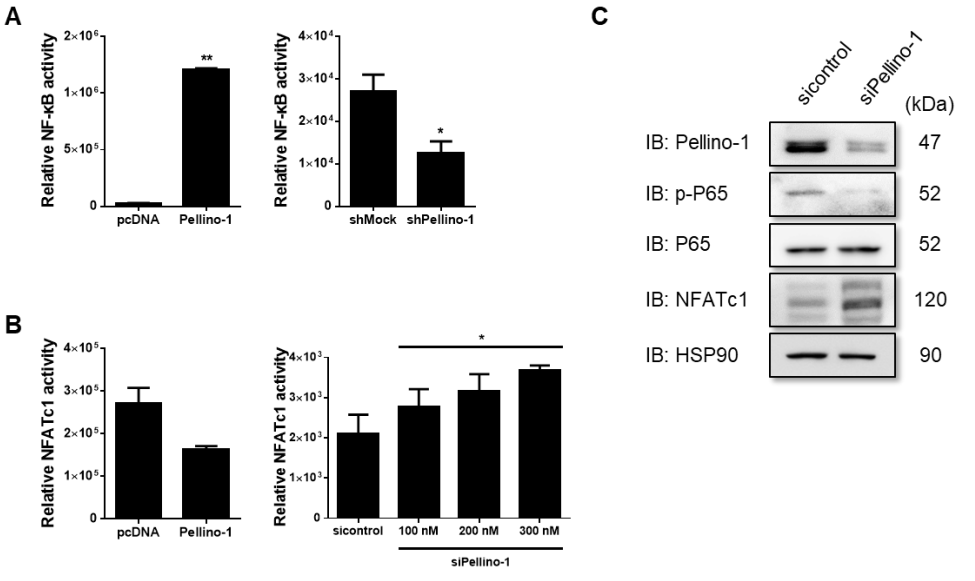

Figure 10. The main transcription factor of the osteoclast is affected by the Pellino-1 modulation. (A, B) Relative promoter activity of NF-kB and NFATc1 after transfected Pellino-1 or siPellino-1 in HEK 293T. Relative luciferase activity was normalized to Renilla. *, $P < 0.05$; **, $P < 0.01$. (C) Protein levels of NF-kB and NFATc1 after transfected siPellino-1 were immunoblotted with the indicated antibodies. All error bars indicate S.D. (* $P < 0.05$, ** $P < 0.01$).

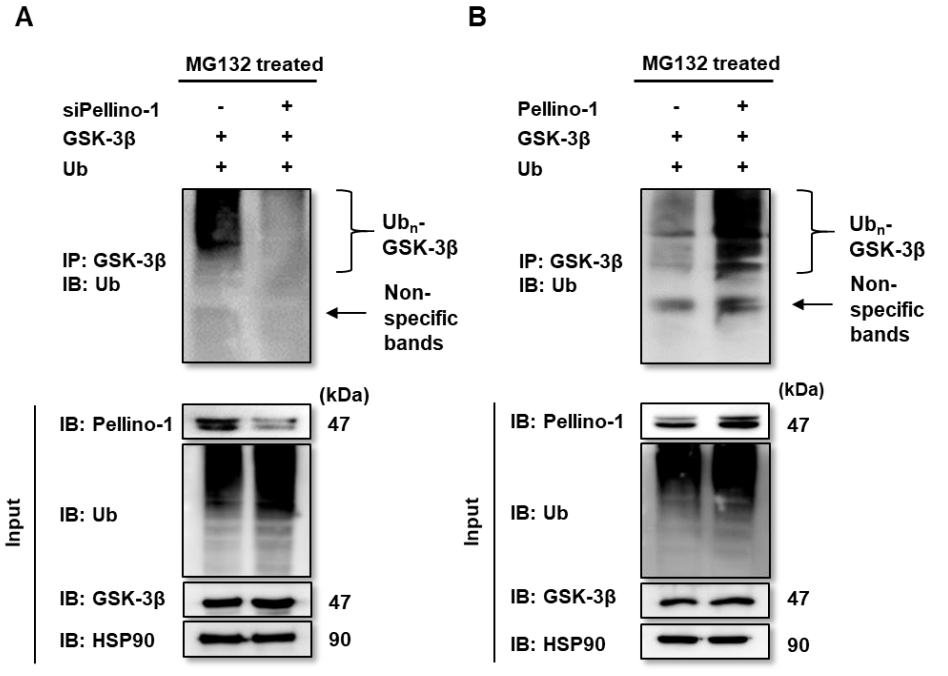

Figure 11. Pellino-1 enhances ubiquitination of GSK-3β (A, B) HEK 293T were transfected with siPellino-1, Pellino-1, along with control vector, ubiquitin, and GSK-3β. MG132 (1 μM) was treated for 24 hours. Cell lysates were subjected to immunoprecipitation with anti- GSK-3β antibody, followed by immunoblotting with anti-ub antibody. (A) Pellino-1 knockdown decreased ubiquitination of GSK-3β. (B) Pellino-1 overexpression increased ubiquitination of GSK-3β.

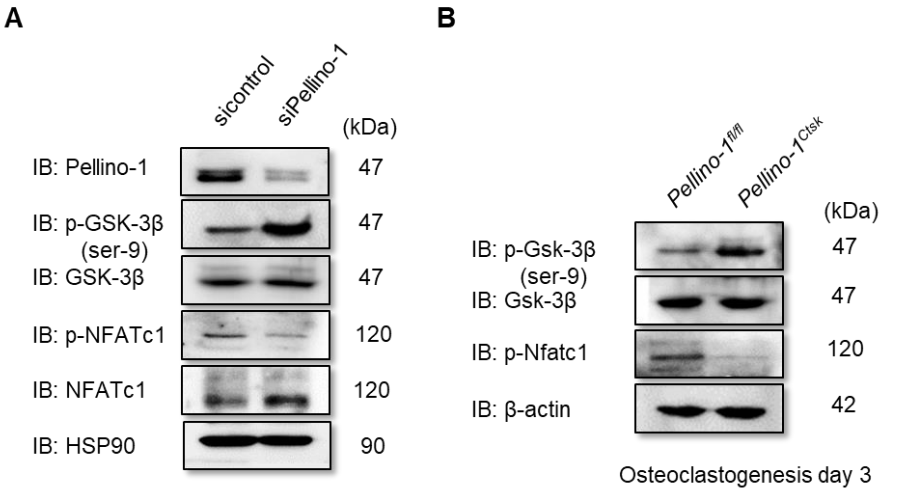

Figure 12. Knockdown of Pellino-1 upregulates phosphorylation of GSK-3β.

(A) Protein levels of indicated genes after transfected siPellino-1 were immunoblotted in HEK 293T. (B) Protein levels of indicated genes from mBMMs isolated from *Pellino-1*$^{fl/fl}$ and *Pellino-1*^{Ctsk} were cultured with 10 ng/ml of M-CSF and 10 ng/ml of RANKL and harvested at 3rd day after osteoclast induction.

To examine that augmented phosphorylation of GSK-3β contributed to excessive osteoclast differentiation through upregulated NFATc1, I transfected siGsk-3β to reduce the total expression of Gsk-3β so that inhibits phosphorylation of Gsk-3β. Knockdown of Gsk-3β rescued phosphorylation of Gsk-3β mBMMs of *Pellino-1*^{Ctsk} (Fig. 13B). Dephosphorylated Nfatc1 translocates to the nucleus[29]. Phosphorylated Nfatc1 was decreased in *Pellino-1*^{Ctsk} and rescued in *Pellino-1*^{Ctsk} transfected siGsk-3β (Fig. 13B). These results demonstrated that the interaction of Pellino-1 and GSK-3β is important in osteoclastogenesis.

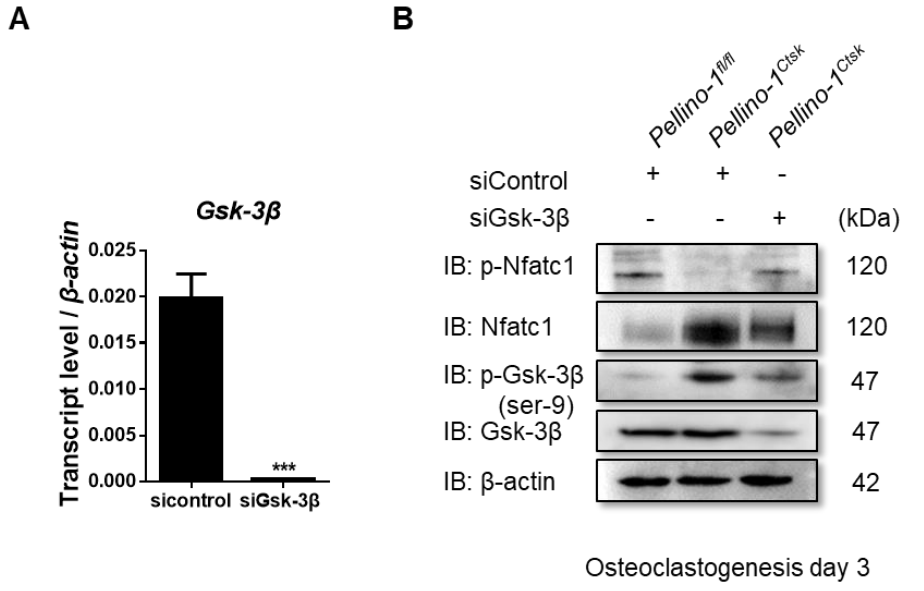

Figure 13. Knockdown of GSK-3β attenuates the NFATc1 activity of Pellino-1 deficient mBMMs. (A) mRNA expression of *Gsk-3β* was decreased when transfected siGsk-3β. (B) Protein levels of indicated genes from mBMMs isolated from *Pellino-1*$^{fl/fl}$ and *Pellino-1*^{Ctsk} after transfected siGsk-3β were immunoblotted. Cells were cultured with 10 ng/ml of M-CSF and 10 ng/ml of RANKL and harvested at 3rd day after osteoclast induction. All error bars indicate S.D. (*** $P < 0.001$).

IV. DISCUSSION

Osteoporosis, characterized decreased bone density with reduced osteoblast numbers, but increased osteoclast numbers, suggesting that it is important to balance osteoclastogenesis and osteoblastogenesis for maintain bone structure and volume. Bone homeostasis is closely connected with each other, suppressing one side will suppress the other side. It is important to develop a treatment that attenuates imbalance of bone coupling process.

Signaling pathways of osteoclastogenesis needs TRAF6 complex to transcript osteoclast marker genes. The activation of osteoclast-related genes requires the recruitment of TRAF6, which is responsible for osteoclast differentiation and activation[30,31]. Pellino-1 ubiquitinates TRAF6 to recruit TRAF6 signaling complex under inflammatory environment[14]. Although Pellino-1 would engage with activation of osteoclast differentiation, Pellino-1 related in osteoclastogenesis has never been discussed before. In this present study, I focused the role of Pellino-1 during osteoclastogenesis.

First, to asses if Pellion-1has a role in the process of osteoclast differentiation, mBMM was induced osteoclastogenesis. Pellino-1 was significantly decreased during the period of osteoclast differentiation (Fig. 1 C, D). Because it is well known that Pellino-1 facilitated the formation of TRAF6 complex, I thought Pellino-1 expression also increased during osteoclastogenesis, it showed an unexpected decreasing pattern. This results suggests Pellino-1 negatively regulates osteoclast differentiation in mBMMs. The other family of Pellino proteins,

Pellino-2 and Pellino-3, also decreased during osteoclastogenesis, but Pellino-1 exhibited predominant basal level and downtrend. It means Pellino-1 is the major regulator of osteoclast differentiation.

Based on prior data, I made conditional knockout mouse model to determine whether the loss of Pellino-1 promotes osteoclastogenesis (Fig. 2). From the 5-week-old, the weight of *Pellino-1*^{ctsk} decreased compared to the wild type group (Fig. 3A) and the body size and femur size was smaller in *Pellino-1*^{ctsk} mice (Fig. 3B, C). However, there is no difference in new born mice, it means that osteoclast activation by Pellino-1 knockout occurs during the postnatal bone development process (Fig. 3D). Furthermore, since osteoclast absorbs mineralized cartilage and hypertrophic chondrocytes[32,33], Pellino-1 could play a crucial role during ossification process. Trabecular bone and cortical bone mass are dramatically declined in *Pellino-1*^{ctsk} mice (Fig. 4, 5). Multi nuclear site and TRAP positive area, the characteristic of osteoclast, were increased in *Pellino-1*^{ctsk} (Fig. 6A, B) and the serum level of CTX-I also upregulated (Fig. 7), suggesting that *Pellino-1* knockout improved osteoclast differentiation. Osteoclastogenesis in *Pellino-1* depletion mBMMs also promotes osteoclast differentiation in *ex vivo* (Fig. 8). Osteoclast marker genes also upregulated in *Pellino-1* depletion mBMMs (Fig. 9). c-Fos induces NFATc1 expression through binding to the NFATc1 promoter[34]. Thus, both NFATc1 and c-Fos were found to have increased in Pellino-1 knockout mBMMs (Fig. 9B). These findings indicated that Pellino-1 is a negative regulator in osteoclastogenesis

On both *in vivo* and *ex vivo*, I identified phenotype that the knockout of Pellino-1 causes excessive osteoclast activity, then I performed the following experiments to find the mechanism for it. NFATc1 is a master regulator of osteoclast differentiation[35,36]. NFATc1 expression is induced during osteoclast differentiation and almost transcription factors of osteoclast marker genes have been found to bind to NFATc1 promoter during osteoclastogenesis. The activation of NFAT is mediated by a specific phosphatase, calcineurin, which is activated by calcium/calmodulin signaling[37]. Interestingly, I confirmed that knockout of Pellino-1 increased NFATc1 transcriptional activity and protein expression (Fig. 10B, C). This data suggested that Pellino-1 suppresses osteoclast differentiation by regulating NFATc1. NFAT-binding sites in the promoter sites of genes like Cathepsin K, osteoclast-associated receptor (OSCAR), which are key regulator of osteoclast differentiation[38]. And knockout of NFATc1 shows fail to differentiate into osteoclasts under stimulation of RANKL[39]. Therefore, NFATc1 is the indispensable regulator of osteoclastogenesis.

I further investigate the mechanism in which Pellino-1 regulates osteoclastogenesis via NFATc1 signaling pathway. GSK-3β is acting as a negative regulator of osteoclast differentiation. After treatment of RANKL, as Ser-9 of GSK-3β becomes phosphorylated, it becomes inactive formation, which helps NFATc1's nuclear location[40,41]. Ubiquitination is a post-translational modifications controlling a variety of intracellular signaling events[42], of course, osteoclast differentiation process needs ubiquitination. Itch interacts with the

deubiquitinating enzyme, CYLD, and is required for deubiquitination of TRAF6, thus limiting RANKL-induced osteoclast formation[43,44]. In late stage of osteoclastogenesis, Cbl-b ubiquitinates and degrades NFATc1 proteins under a Src-dependent manner[34]. Because Pellino-1 is an E3 ligase, I conducted a ubiquitination assay to confirm whether Pellino-1 regulates GSK-3β's activity by ubiquitination. An *in vitro* ubiquitination assay revealed that GSK-3β ubiquitination was strikingly inhibited by knockdown of Pellino-1 (Fig. 11A). In contrast, overexpression of Pellino-1 dramatically increased GSK-3β ubiquitination (Fig. 11B). Implying that the ubiquitination of GSK-3β is depend on Pellino-1 and it could make activated condition of GSK-3β. Then, I confirmed that knockdown of Pellino-1 increased phosphorylation on serine 9 of GSK-3β (Fig. 12A). Under RANKL stimulation in mBMMs, it showed corresponding results to the previous one and decreased phosphorylation of Nfatc1 indicated that Nfatc1 is activated so that translocated into nuclear (Fig. 12B). Taken together, Pellino-1 regulates GSK-3β's activity and NFATc1's activity.

If GSK-3β phosphorylation is increased by deletion of Pellino-1, I checked whether excessive NFATc1 expression can be rescued due to reduced Pellino-1/ GSK-3β axis activity by silencing GSK-3β. As a result, when the total amount of Gsk-3β was knocked down, the phosphorylation was also reduced, resulting in a decrease in Nfatc1 compared to mBMMs of *Pellino-1*$^{fl/fl}$ transfected sicontrol. (Fig. 13B). Nfatc1, which increased excessively as Pellino-1 was knocked out, was found to decrease with GSK-3β silencing. These findings suggest that Pellino-1

plays a significant role in activation of NFATc1 via Pellino-1/GSK-3β axis in RANKL-mediated osteoclast differentiation.

Collectively, Pellino-1 negatively regulates osteoclast differentiation through activation of GSK-3β and suppression of NFATc1.

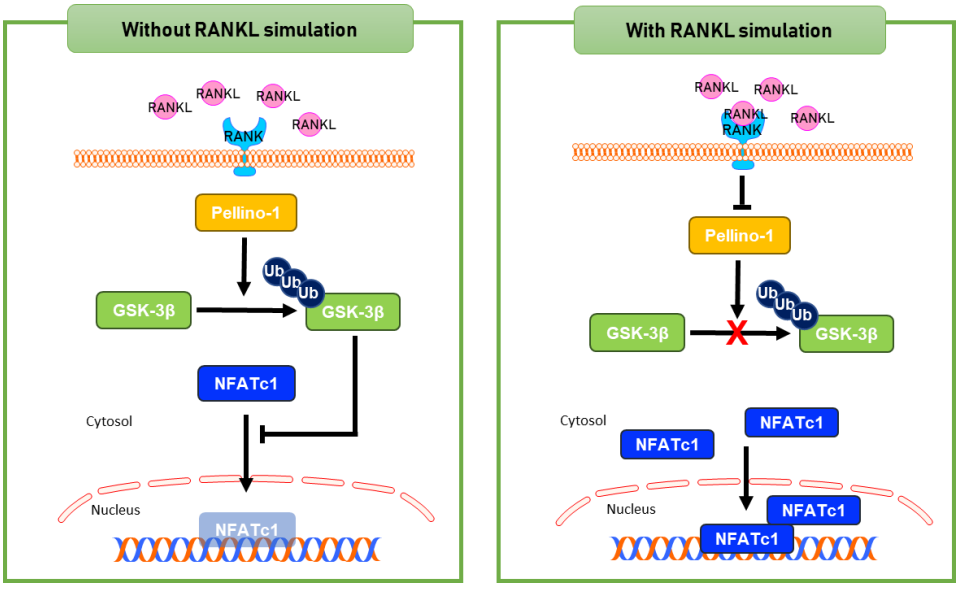

Figure 14. Scheme of showing the molecular mechanism. A diagram showing the molecular mechanism in which Pellino-1 promotes osteoclast differentiation. Without RANKL stimulation, Pellino-1 induces ubiquitination of GSK-3β so that GSK-3β negatively regulates NFATc1. With RANKL stimulation to initiate osteoclastogenesis, the expression of Pellino-1 is decreased. Accordingly, ubiquitination of GSK-3β is down-regulated and transcription of NFATc1 is activated.

V. CONCLUSION

In this study, I demonstrated that the Pellino-1 acts as a negative regulator of osteoclast differentiation. During osteoclastogenesis, expression of Pellino-1 was decreased, and bone mass declined due to the excessive bone resorption in conditional knockout of Pellino-1 in osteoclasts. Knockout of Pellino-1 in osteoclasts also increased osteoclast differentiation and the secretion of the bone resorption marker CTX-1. Through luciferase analysis, knockdown of Pellino-1 increased transcriptional activity of NFATc1, the master regulator of osteoclast differentiation. Furthermore, Pellino-1 promotes ubiquitination of GSK-3β. Overall, Pellino-1 activates GSK-3β activity so that this Pellino-1 could suppress NFATc1. For these reasons, Pellino-1 will play an important role as a cell therapy for osteoporosis by targeting GSK-3β.

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ABSTRACT (IN KOREAN)

파골세포 분화 기간 동안 NFATc1 신호 조절 인자로서 Pellino-1의 역할

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정 수 진

뼈의 항상성은 조골세포에 의한 골형성과 파골세포에 의한 골흡수의 적절한 균형에 의해 조절된다. 이 과정의 조절 실패는 골다공증과 같은 질환을 유발한다. 많은 문헌에서 파골세포의 분화과정에 관련된 다양한 신호전달 체계가 보고되고 있으며, E3 연결 효소인 Pellino-1은 다양한 표적 단백질들의 유비퀴틴화를 통해 세포 신호 전달 체계를 조절한다고 알려져 있다. 따라서 본 연구에서는 파골세포 분화에 있어서 Pellino-1의 기능 및 신호전달 기전을 밝히고자 하였다.

Pellino-1 유전자의 결손이 마우스 파골세포의 분화와 골흡수 표지인자인 CTX-1의 분비를 증가시켰으며, 골밀도를 감소시킴을 동물실험에서 확인하였다. 파골세포 분화 과정 중 Pellino-1의 작용 기전을 규

명하기 위해 Pellino-1을 억제하였을 때 NFATc1의 프로모터 활성은 증가하였고, 불활성화된 GSK-3β의 발현이 증가하였다. 또한, 유비퀴틴화 분석을 통해 Pellino-1이 GSK-3β의 유비퀴틴화를 유도함을 확인하였다. 이러한 결과들은 파골세포 분화 과정에서 Pellino-1이 GSK-3β의 활성을 조절함을 의미한다. 이를 통해, 파골세포에서 Pellino-1의 결손이 GSK-3β를 불활성화 시킴으로써 파골세포 분화를 증가시키는 것을 확인 하였다. 따라서 본 연구에서는 파골세포 분화의 억제 조절자로서의 Pellino-1의 기능을 새로이 규명하였으며, 골다공증 및 골절과 같은 골 리모델링 관련 골격 질환의 치료 표적이 될 수 있을 것이라 기대한다.

핵심되는 말: Pellino-1, 골 항상성, 파골세포 분화, 골다공증, NFATc1, GSK-3β