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THOC5 regulates human osteoclastogenesis

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Abstract

Osteoclasts are bone resorbing cells that are responsible for physiological and pathological bone resorption. Macrophage colony stimulating factor (M-CSF) binds to the M-CSF receptor (c-FMS) and plays a key role in the differentiation and survival of macrophages and osteoclasts. THOC5, a member of the THO complex, has been shown to regulate hematopoiesis and M-CSF-induced macrophage differentiation. However, the role of THOC5 in osteoclasts remains unclear. Here, our study reveals a new role of THOC5 in osteoclast formation. We found that THOC5 shuttles between nucleus and cytoplasm in an M-CSF signaling dependent manner. THOC5 bound to FICD, a proteolytic cleavage product of c-FMS, and THOC5 facilitates the nuclear translocations of FICD. Decreased expression of THOC5 by siRNA-mediated knock down suppressed osteoclast differentiation, in part, by regulating RANK, a key receptor of osteoclasts. Mechanistically, knock down of THOC5 inhibited the expression of RANKL-induced FOS and NFATc1. Our findings highlight THOCS′s function as a positive regulator of osteoclasts.

Keywords

Osteoclasts; THOC5; M-CSF; C-FMS

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CRediT authorship contribution statement

Se Hwan Mun: Conceptualization, Investigation, Data curation, Writing – original draft, Visualization. **Brian Oh**: Investigation, Data curation, Writing – review & editing, Visualization. **Min Joon Lee**: Data curation. **Seyeon Bae**: Data curation. **Young Yang**: Supervision, Funding acquisition. **Kyung-Hyun Park-Min**: Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejcb.2022.151248.

Declaration of Competing Interest

The authors declare no conflicts of interest.

1. Introduction

Osteoclasts are the sole bone resorbing cells (Teitelbaum, 2000; Tsukasaki and Takayanagi, 2019) and are derived from myeloid lineage precursor cells (Jacome-Galarza et al., 2013; Xiao et al., 2015). Macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB (RANKL) are key factors for osteoclast differentiation (Park-Min, 2018; Teitelbaum, 2000). M-CSF is a ligand for c-FMS (also known as CSF1R), and M-CSF binding to c-FMS induces a receptor dimerization and autophosphorylation of c-FMS that leads to activation of canonical M-CSF signaling pathways(Mun et al., 2020; Ross, 2006). M-CSF signaling has been shown to support survival of osteoclast precursor cells (OCPs) and promote the expression of RANK, a receptor for RANKL. RANKL signaling induces the activation of MAP kinases and NF-kB, resulting in the induction of FOS, MYC, and NFATc1, key regulators of osteoclasts (Bae et al., 2017; Grigoriadis et al., 1994; Takayanagi et al., 2002).

THOC5 is a member of the THO (suppressor of delta hpr1 transcriptional defects by overexpression) complex (Tran et al., 2016). The THO complex consists of core subunits such as THOC1, THOC2, THOC3, THOC5, THOC6, and THOC7, and additional proteins. The THO complex is a subcomplex of the transcription/export multiprotein complex (TREX) that plays a role in mRNA export from the nucleus, transcriptional elongation, and genome stability (Jimeno et al., 2002; Masuda et al., 2005; Rehwinkel et al., 2004; Wang et al., 2013). THOC5 (also known as Fms interacting protein, FMIP) transiently binds to c-FMS and is also a substrate of c-FMS (Tamura et al., 1999) and other tyrosine kinases(Pierce et al., 2008). THOC5-deficiency is an embryonically lethal phenotype in mice(Mancini et al., 2010). THOC5 deficiency regulates self-renewal and differentiation of embryonic stem cells, somatic cell reprogramming and blastocyst development by regulating import of pluripotency gene transcripts (Wang et al., 2013). Consistently, THOC5 inducible deletion leads to death in mice within 14 days (Mancini et al., 2010) but affects the processing of only less than 1% of transcripts (Saran et al., 2013), suggesting the existence of multiple functions of THOC5, in addition to mRNA export. THOC5 also has been shown to be involved in the differentiation of various cell types including hematopoietic stem cells, adipocytes, and mesenchymal stem cells (Tran et al., 2016). THOC5 also prevents apoptosis by regulating inositol lipid turnover (Pierce et al., 2008). THOC5 gene polymorphisms are correlated with lipid and metabolic parameters in middle aged women (Loja-Chango et al., 2020). However, the underlying mechanism of THOC5′s actions is still poorly understood.

The role of THOC5 in osteoclast differentiation remains unclear. Here, we identified a novel role of THOC5 in human osteoclastogenesis. Upon M-CSF stimulation, THOC5 shuttled between the cytoplasm and the nucleus in human OCPs and blocking M-CSF signaling retained THOC5 in the cytoplasm. In addition to c-FMS, THOC5 also interacted with FMS intracellular domains (FICDs), recently identified cleavage products of c-FMS (Mun et al., 2021). Knock-down of THOC5 in human OCPs using small interfering RNAs (siRNAs) diminished the nuclear translocation of FICDs and suppressed osteoclastogenesis. Mechanistically, decreased expression of THOC5 suppressed RANKL-induced NFATc1 expression. Our study provides insights into the role of THOC5 in cellular differentiation in human osteoclasts.

2. Materials and methods

2.1. Cells

Peripheral blood mononuclear cells (PBMCs) from blood leukocyte preparations purchased from the New York Blood Center were isolated by density gradient centrifugation with Ficoll (Invitrogen, Carlsbad, CA). $CD14^+$ cells were obtained by positive selection using anti-CD14 magnetic beads (Miltenyi Biotec, CA), following the manufacturer's protocols. Human $CD14^+$ cells were cultured in α-MEM medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone; SH30070.03) and 1% L-glutamine with 20 ng/ml of M-CSF for 12 h to generate osteoclast precursor cells (OCPs). The purity of monocytes was > 97%, as verified by flow cytometric analysis (Park-Min et al., 2014). Human osteoclastogenesis assays were performed by a modification of previously published methods (Park-Min et al., 2014). Briefly, siRNA transfected $CD14^+$ cells were added to 96 well plates in triplicate at a seeding density of 5×10^4 cells per well. Osteoclast precursors were incubated with 20 ng/ml of M-CSF and 40 ng/ml of human soluble RANKL for 3 days. Cytokines were replenished every 3 days. Cells were fixed and stained for TRAP using the Acid Phosphatase Leukocyte diagnostic kit (Sigma; 387 A) as recommended by the manufacturer. Multinucleated (greater than 3 nuclei), TRAP-positive osteoclasts were counted in triplicate wells.

2.2. Immunoblot

Whole cell extracts or nuclear lysates were prepared as previously described (Mun et al., 2021). The protein concentration of nuclear extracts was quantitated using the Bradford assay (Bio-Rad; 5000001). Total, cytoplasmic or nuclear proteins were separated on 7.5 or 10% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes (PVDF, Millipore; ISEQ00010), and detected by antibodies as listed in Table S1.

2.3. RNA preparation and real-time PCR

DNA-free RNA was obtained using the RNeasy Mini Kit from QIAGEN with DNase treatment, and 0.5 μg of total RNA was reverse transcribed using a First Strand cDNA Synthesis kit (Fermentas, Hanover, MD). Real time PCR was performed in triplicate using the iCycler iQ thermal cycler and detection system (Applied Biosystems, Carlsbad, CA) as recommended by the manufacturer. The primer sequences are listed in Table S2.

2.4. RNA Interference

RNA interference 0.2 nmol of small interfering RNAs (siRNAs), specifically targeting human THOC5 (Dharmacon, J-015317–09–0002 and J-015317–12–0002) or control siRNA (Dharmacon, D-001810–10) were transfected into primary human $CD14^+$ monocytes with the Amaxa Nucleofector device, set to program Y-001 using the Human Monocyte Nucleofector kit, (Amaxa), as previously described (Mun et al., 2021).

2.5. Immunoprecipitation

293 T cells were transfected with pCMV6-Entry-c-FMS-MYC-DDK (NM_005211, Origene, Rockville, MD) using Lipofectamine 3000 (ThermoFisher scientific) as previously

described (Mun et al., 2021). Transfected cells were incubated with M-CSF (20 ng/ml) for one day, and nuclear proteins were immunoprecipitated (IP) with antibodies against the N-terminal region of c-FMS (Santa Cruz; H-300 and R&D systems; clone #61780) to remove full-length c-FMS as a negative selection. Subsequently, the IP-proteins were incubated with either mouse IgG or DDK-tag Ab conjugated magnetic beads (Origene). Proteins bound to ab-beads were eluted with water. Samples were subjected to SDS PAGE gel and were analyzed by immunoblotting using anti-THOC5 antibodies. Human $CD14^+$ monocytes or siRNA transfected $CD14^+$ cells were seeded into 100 mm dishes and incubated with M-CSF (20 ng/ml) for 48 h. Cells were lysed with RIPA buffer with a proteinase inhibitor cocktail. An equal amount of cell lysates was prepared and incubated with Agarose A/G beads (Thermo Scientific), IgG (Santa Cruz, sc-2027), anti-c-FMS antibody (Santa Cruz, sc-962), or anti-THOC5 antibody (Bethyl Laboratories, A032–120A) for 24 h at 4 °C. The beads were washed 5 times with washing buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% NP-40, 1% glycerol, protease and phosphatase inhibitors). Proteins eluted from the bead with elution buffer (pH 2.8, Prod#1858606). The samples were incubated in 95 °C for 10 mins and then were analyzed by immunoblotting.

2.6. CCK8 analysis

To measure cell activity, the Cell Counting Kit-8 assay (CCK8; Dojindo Laboratories) was used. After transfection, cells were seeded in 96-well plates in the presence of M-CSF with or without RANKL, and analysis was performed as previously described (Bae et al., 2021). Absorbance at 450 nm was monitored during a period of 4 h following treatment with the assay reagent.

2.7. Bone resorption pit assay

After nucleofection of siRNAs, osteoclast activity in vitro was measured with a bone resorption assay. Human CD14 + cells were seeded a density of 2×10^5 cells per well in a 48-well Bone Resorption Assay Plate (Cosmo Bio USA) and cultured in the presence of M-CSF and RANKL. After verifying the presence of osteoclasts and pit formation, the cells were removed and wells were subsequently stained with toluidine blue solution as described in Mun et al. (2021). The total area of the pits was measured using ImageJ with the Fiji plugin package.

2.8. Immunofluorescence microscopy

Human $CD14^+$ macrophages were starved for three hours and then were cultured with M-CSF (20 ng/ml) in a culture slide (SLP; 30108) for 5 days. Cells were fixed with 3.7% formalin in PBS for 20 min at room temperature at the indicated time points. Cells were permeabilized with 1% triton X-100 for 5 mins and were then washed 3 times before blocking with solution that contains 5% FBS and 5% BSA (without IgG) in PBS for 1 h. Slides were subsequently incubated with anti-THOC5 antibodies (SantaCruz Biotechnology; sc-514146) overnight at 4 °C, followed by incubation with FITC-conjugated goat anti-mouse IgG antibodies (Bethyl; A90–105 F) for 40 min at room temperature. After washing, cell nuclei were stained with ProLongTM Gold antifade regent with-DAPI (P36931, Invitrogen). Images were captured using a Zeiss Axioplan microscope (Carl Zeiss) with an attached Leica DC 200 digital camera (Leica) or a confocal microscope system

(Carl Zeiss LSM 700, Laser excitation/emission: 405/425 and 488/525). To examine THOC5 in the nucleus, confocal three-dimensional Z-stacks were acquired for each sample using a Plan-Apochromat 63 × /1.4 water Dic M27 objective (Zeiss, Germany) with a slice of increment of 0.9 μm. The images were processed with ZEN lite (Carl Zeiss) software as previously described (Mun et al., 2021).

2.9. Statistical analysis

GraphPad Prism 8.0 was used for all statistical analysis. Detailed information about statistical analysis, including tests and values used and number of times experiments were repeated, is provided in the figure legends. A *p*-value less than 0.05 was statistically significant. We performed the non-parametric Kolmogorov-Smirnov test for two conditions. For more than two conditions, Shapiro-Wilk normality tests were performed; for data that fell within a Gaussian distribution we performed appropriate parametric statistical tests and for those that did not fall within an equal variance-Gaussian distribution, we performed the Friedman test, a non-parametric statistical test.

3. Results

3.1. THOC5 dynamically shuttles between nucleus and cytoplasm by c-Fms signaling

To examine the role of THOC5 in human osteoclast precursor cells (OCPs), we measured the expression of THOC5. THOC5 expressed and was constantly maintained in human OCPs for up to 48 h after M-CSF treatment (Fig. 1A). Since THOC5 has been shown as a shuttle protein responsible for transporting mRNA and nuclear proteins without nuclear localization signals (Carney et al., 2009), we wished to determine the localization of THOC5. THOC5 expression was detected both in the nucleus and the cytoplasm after M-CSF stimulation (Fig. 1B). The levels of cytosolic THOC5 were dynamically regulated and were reduced to the levels of no-M-CSF conditions at 48 h after M-CSF simulation (Fig. 1B). Conversely, THOC5 in the nucleus was diminished at 24 h after M-CSF stimulation but increased at 48 h after M-CSF stimulation. To further test whether localization of THOC5 was regulated by c-FMS signaling, human OCPs were treated with Imatinib, a tyrosine receptor inhibitor, to block c-FMS activation prior to M-CSF stimulation (Druker et al., 2001). Imatinib treatment reversed the increase of THOC5 in the nuclear fraction at 48 h after M-CSF stimulation (Fig 1C and D). To further examine the subcellular distribution of THOC5, cells were cultured with M-CSF for 5 days and THOC5 expression was determined using immunofluorescence microscopy. THOC5 was mainly detected in the nucleus on day 2 and then diffused into both nucleus and cytoplasm on day 3. THOC5 was also localized in a vesicle-like structure in the cytoplasm on day 5. Our data suggests that THOC5 dynamically shuttles between the nucleus and the cytoplasm in a M-CSF signaling-dependent manner.

3.2. THOC5 facilitates the translocation of FICD into the nucleus

THOC5 has been shown to transiently interact with c-FMS (Tamura et al., 1999). We have shown that c-FMS undergoes a series of proteolysis upon M-CSF stimulation and generates c-FMS intracellular cytoplasm domains (FICDs) (Mun et al., 2021). Moreover, FICDs were translocated into the nucleus after M-CSF stimulation and nuclear FICD was named L-FICD

(Fig. S1). To determine whether THOC5 interacts with FICDs in the nucleus, we performed immunoprecipitation assays. Nuclear proteins were isolated from c-FMS overexpressed 293 T cells and were subjected to immunoprecipitation with anti-c-Fms antibodies. THOC5 was detected by immunoblot (Fig. 2A), suggesting that nuclear FICDs interact with THOC5. To corroborate our findings, we next wished to determine whether THOC5 interacts with endogenous FICDs in human OCPs. Cytoplasmic and nuclear proteins were subjected to immunoprecipitation with anti-c-Fms antibodies and THOC5 expression was determined by immunoblot. Intriguingly, THOC5 was detected mainly in the nuclear fractions, although mature (M) and immature (I) c-FMS were mainly located in the cytoplasmic fractions. Our results indicated that THOC5 bound to FICD in the nucleus at 48 h after M-CSF stimulation (Fig. 2B). FICDs do not contain a nuclear localization signal (Lusk et al., 2007), and how FICDs translocate into the nucleus remains unclear. Thus, to test whether THOC5 facilitated the nuclear translocation of FICDs, we knocked down the expression of THOC5 in human OCPs using small interfering RNAs (siRNAs). Strikingly, the nuclear localization of FICDs was diminished in THOC5 KD cells compared to control cells. (Fig. 2C). Our data suggests that the THOC5-FICD complex is translocated into the nucleus and THOC5 may function as a shuttle protein of FICDs.

3.3. THOC5 regulates human osteoclast formation

Both c-FMS and FICD play an important role in osteoclast differentiation (Mun et al., 2021, 2020). We next tested if THOC5 contributes to osteoclast formation. To do so, we first measured the expression of THOC5 during osteoclastogenesis. Human OCPs which were generated from CD14 + cells after M-CSF stimulation were cultured with M-CSF and RANKL for additional three days. While THOC5 mRNA expression was minimally affected by RANKL stimulation, nuclear THOC5 was induced by RANKL stimulation in a time-dependent manner (Fig. 3A and B). The expression of THOC5 was silenced by nucleofecting THOC5 siRNAs. Control (NC) and THOC5 knock-down (KD) cells were then cultured with M-CSF (20 ng/ml) for one day and then were stimulated with M-CSF and RANKL (40 ng/ml) for an additional three days. THOC5 expression was efficiently diminished by THOC5 knock-down (Fig. 3C and D). However, the silencing of THOC5 expression had no effect on cell viability in human osteoclasts (Fig 3E) and macrophages (Fig. S4 A). Decreased expression of THOC5 resulted in decreased osteoclast formation by 45%, compared to control cells (Fig. 3F) and a trend toward to diminished bone resorption (Fig. S3). Accordingly, mRNA expression of osteoclast specific genes such as Calcitonin receptor (CTR) and Cathepsin K (Cat-K) were suppressed in THOC5 KD cells relative to control KD cells (Fig. 3G). Thus, our data implicates THOC5 as a positive regulator of osteoclastogenesis.

NFATc1 is a master regulator of osteoclastogenesis (Takayanagi et al., 2002). To elucidate the underlying mechanism by which THOC5 regulates osteoclasts, we measured RANKL-induced NFATc1 and c-Fos by immunoblot in control and THOC5 KD cells. Induction of both NFATc1 and c-Fos protein was diminished in THOC5 KD cells compared to control cells (Fig. 4A). Accordingly, RANKL-induced NFATc1 mRNA levels were significantly reduced in THOC5 KD cells relative to control cells (Fig. 4B). RANK, a receptor for RANKL, is an upstream regulator of NFAT1c1, and was induced by M-CSF signaling

(Fig. S4B). We measured the expression of RANK in human OCPs. RANK expression was down-regulated in THOC5 KD cells compared to control cells, while the levels of c-FMS were comparable between THOC5 KD cells and control cells (Fig. 4C and D, Fig. S4B). Taken together, our data indicate that THOC5 may be involved in RANK expression, thereby regulating NFATc1 expression and osteoclastogenesis.

4. Discussion

M-CSF signaling plays a critical role not only in macrophage differentiation but also in osteoclast formation (Mun et al., 2020; Ross, 2006). Osteoclasts are the sole bone resorbing cells and play a key role in bone homeostasis. The mechanisms of canonical M-CSF signaling pathways in osteoclasts are well defined, and increased expression of M-CSF is correlated with several pathological conditions such as rheumatoid arthritis (Paniagua et al., 2010). We have shown that THOC5 positively regulates human osteoclast differentiation and is associated with FICDs. THOC5 knock down suppressed the expression of RANKL-induced NFATc1, a master regulator of osteoclastogenesis.

Our study reveals a novel role of THOC5 in osteoclast differentiation. THOC5 deficiency resulted in defects in hematopoiesis and alteration of other cell types (Mancini et al., 2010; Tran et al., 2016). Overexpression of THOC5 in FDC-P1Mac1, a myeloid progenitor cell line, inhibited M-CSF induced macrophage differentiation and skewed cells to differentiate into granulocytes. Previous studies suggested that THOC5 provides a threshold for myeloid cell differentiation. However, the role of THOC5 in osteoclast formation has not been previously shown. Our previous study demonstrated that human osteoclasts that differentiated from human $CD14^+$ cells can resorb bone and were functionally active (Park-Min et al., 2014). We also found that during osteoclastogenesis, THOC5 levels in the nucleus were continuously induced, which contrasts with the dynamic shuttling of THOC5 in macrophages. Moreover, THOC5 knockdown did not alter cell viability in nonproliferating human macrophages and osteoclasts. RANKL, a ligand for RANK, is a key driver of osteoclast differentiation; our study showed that THOC5 regulates osteoclastogenesis, in part, by affecting *RANK* mRNA expression. However, how THOC5 regulates *RANK* mRNA expression and osteoclastogenesis is incompletely determined. Since M-CSF signaling sharply increased *RANK* mRNA expression in human OCPs, it is possible that decreased THOC5 may attenuate M-CSF signaling, thereby reducing RANK expression. In addition, it has been shown that THOC5 also has the capability to control mRNA export (Tran et al., 2016). Our study also suggests that THOC5 may cargo FICDs and other NLS-less proteins in human OCPs. Thus, these data suggest that the role of THOC5 in osteoclastogenesis may not be limited to the regulation of RANK expression and that THOC5 can regulate osteoclastogenesis in a FICD-dependent manner, as well as in a FICD-independent manner (Fig. 4E).

Our data showed that THOC5 can bind to FICDs. Intriguingly, the interaction between THOC5 and FICDs was detected mainly in the nucleus at two days after M-CSF stimulation; there are several likely explanations. Since both FICDs and small percentages of c-FMS were found in the nucleus, it is possible that THOC5 binds to not only FICDs but also to c-FMS in the nucleus. FICD is the c-terminal domain of c-FMS and contains the verified

binding site of THOC5 (Tamura et al., 1999). NLS in THOC5 is located at aa 7–1,1 and the NLS deletion mutant had 40% of the binding activity to c-FMS relative to control THOC5. Thus, although FICDs did not contain NLS, it is possible that FICDs translocate into the nucleus by binding to THOC5. Tamura et al. detected transient interactions between THOC5 and the c-terminal domain of c-FMS after M-CSF stimulation; THOC5 binds to c-FMS immediately after M-CSF stimulation and is then released at 10 mins after M-CSF stimulation (Tamura et al., 1999), suggesting that the interaction between THOC5 and cytoplasmic FICDs may be transient. We previously showed that cytoplasmic FICDs appeared at 12–18 h after M-CSF stimulation and continuously accumulated, while nuclear FICDs were clearly visible at 48 h after M-CSF stimulation (Mun et al., 2021). Here we showed that the majority of THOC5 was localized in the nucleus at 48 h after M-CSF stimulation, suggesting that the complexes may be preferentially located into the nucleus at 48 h after M-CSF stimulation. Other members of the TREX complexes bind to nuclear FICD, based on our prior mass spectrometry analysis (Mun et al., 2021). These results suggest the possibility that the interaction between THOC5 and FICD in the nucleus can be stabilized by forming bigger complexes by binding to other proteins.

We showed that THOC5 shuttled between the cytoplasm and the nucleus in human OCPs in an M-CSF signaling-dependent manner. It has been shown that subcellular localization of THOC5 shifted during mouse bone marrow derived macrophage differentiation (Tran et al., 2013). On day 4 after macrophage differentiation, THOC5 was mainly located in the nucleus but diffused into the cytoplast on day 8 after differentiation (Tran et al., 2013). Consistently, THOC5 was also detected in the nuclear speckle-like domain of the nucleus on day 2 after replenishing M-CSF in human macrophages. THOC5 then diffused into both the cytoplasm and the nucleus on day 3 and was localized in a vesicle-like structure in the cytoplasm. The nuclear speckles have been shown to serve as sites for storing splicing factors (Lamond and Spector, 2003). However, the functional significance of the sub-localization of THOC5 is poorly explored.

5. Conclusions

Although THOC5 is a known substrate of c-FMS kinase activity (Tamura et al., 1999), c-FMS mediated regulation of THOC5 has been incompletely understood. We show that c-FMS signaling regulated the subcellular localization of THOC5 in human OCPs. Moreover, THOC5 also regulated M-CSF induced *RANK* mRNA expression and suppressed RANKL-induced NFATc1 activation. In addition to c-FMS, THOC5 was capable of binding to FICD and facilitating the nuclear translocation of FICDs. Therefore, our study uncovers a novel role of THOC5 in osteoclastogenesis and the underlying mechanisms concerning THOC5-mediated regulation of osteoclast differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Fig. 4e was generated by Biorender.

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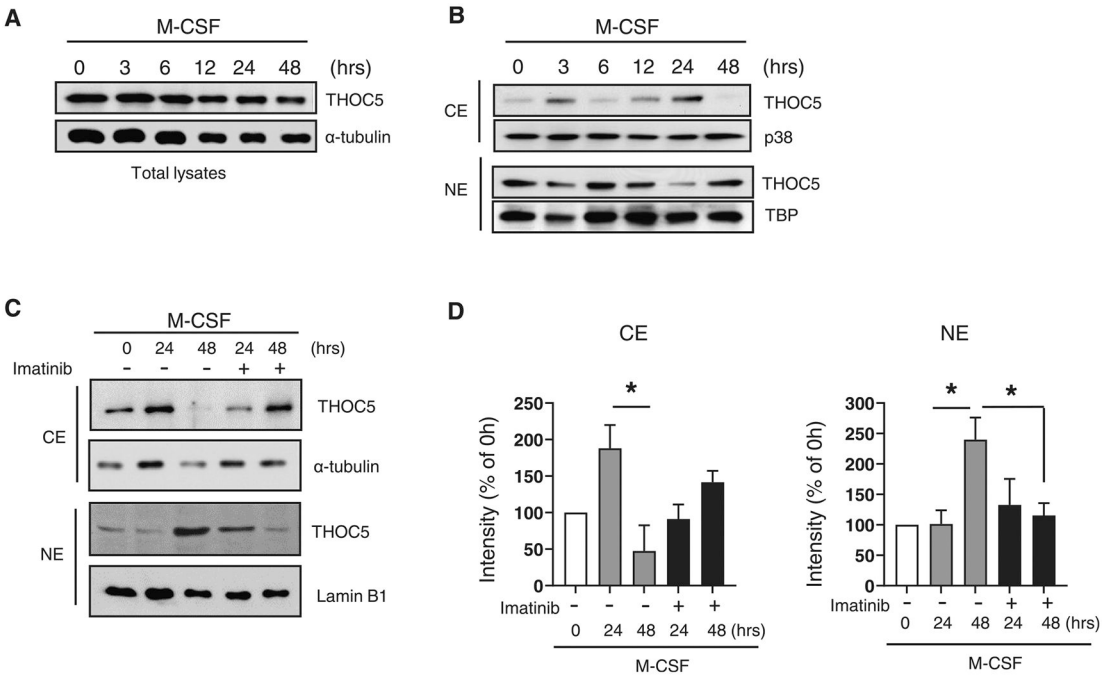

Fig. 1.

Subcellular localizations of THOC5 were dynamically regulated during osteoclastogenesis. Human macrophages were cultured with human M-CSF (20 ng/ml) up to 48 h. (A) THOC5 expression in whole cell lysates for the indicated times (B) Immunoblot analysis of THOC5 in cytoplasmic lysates (CE) and nuclear lysates (NE), prepared from human macrophages with M-CSF at the indicated times. p38 and TBP were used as loading controls for cytoplasmic and nuclear proteins, respectively. (C and D) THOC5, as measured by immunoblot analysis, in human macrophages that were treated with imatinib (0.3 μM) prior to M-CSF treatment. α-tubulin and Lamin B1 were used as controls for the cytoplasmic and nuclear fractions, respectively. Representative images from at least three experiments were shown. (D) Densitometric quantitation of intensity of THOC5 bands, from C, is shown from 3 experiments. The intensity of THOC5 bands in the non-treatment imatinib condition was set as 100% (n = 3). Values represent mean ± SEM. *, $p < 0.05$ by Friedman test (D).

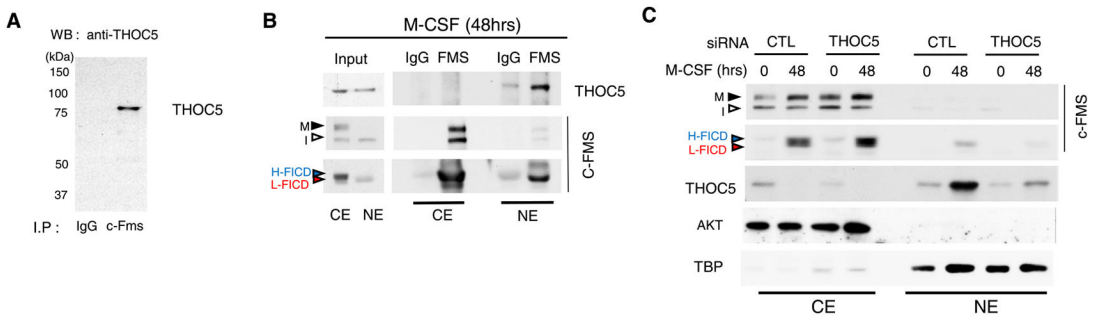

Fig. 2.

THOC5 binds to FICD and enables the translocation of FICD into the nucleus. (A) 293 T cells were transfected with MYC-DDK-double tagged full-length c-FMS and stimulated with M-CSF (20 ng/ml). Overexpressed MYC-DDK-double tagged full-length c-FMS was immunoprecipitated by anti-DDK antibodies from nuclear lysates, and THOC5 was detected by immunoblot with anti-THOC5 antibodies. (B) Human macrophages were cultured with M-CSF for 48 h. Immunoblot analysis of THOC5 in nuclear lysates (NE) and cytoplasmic lysates (CE) after immunoprecipitation with anti-c-FMS or IgG antibodies. Endogenous THOC5 in complex with FICD was detected by immunoblot in human macrophages. (C) Immunoblot analysis. Human monocytes were nucleofected with negative control (CTL) or THOC5-specific small interfering RNAs (siRNAs). Control and THOC5 knockdown OCPs were cultured with M-CSF (20 ng/ml). L-FICD proteins in THOC5 KD cells were unable to translocate into the nucleus. AKT and TBP were used as loading controls for cytoplasmic and nuclear proteins, respectively. M: mature form, I: immature form, H: high mass, L: low mass. Representative images from at least three experiments were shown.

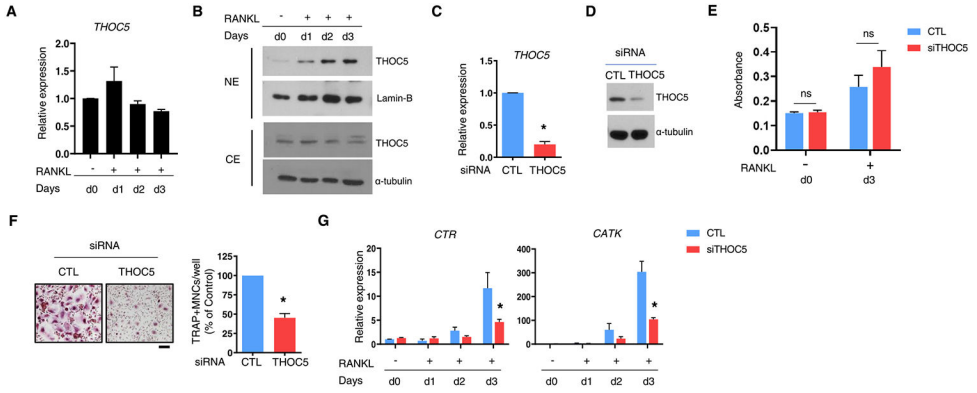

Fig. 3.

THOC5 regulates human osteoclast formation. (A and B) Human CD14 + monocytes were cultured with M-CSF (20 ng/ml) for two days to differentiate into osteoclast precursor cells (OCPs) and then were cultured with M-CSF (20 ng/ml) and RANKL(40 ng/ml) for an additional 3 days. (A) RT-qPCR assay for THOC5 mRNA expression (n = 3). (B) Immunoblot assay for THOC5 protein expression (n = 3). (C to G) Human monocytes were nucleofected with negative control (CTL) or THOC5-specific small interfering RNAs (siRNAs). Negative control and THOC5 KD OCPs were cultured with M-CSF (20 ng/ml) and RANKL (40 ng/ml) for 4 days. (C) THOC5 knock-down efficiency in human OCPs was measured by RT-qPCR (n = 6) and (D) immunoblot assay (n = 3). (E) The viability of cells was analyzed by the CCK8 assay kit (n = 3). (F) Osteoclastogenesis assay. *Left*, TRAP staining of representative wells. Scale bar: 100 μm. *Right*, quantification of TRAP-positive multinucleated (more than three nuclei) cells, normalized relative to the number of osteoclasts formed in control siRNA conditions (n = 4). (G) RT-qPCR analysis of osteoclast specific genes in control and THOC5 KD OCPs, normalized relative to GAPDH mRNA (n = 3). Values represent mean ± SEM. * , $p < 0.05$ by Kolmogorov-Smirnov test (C and F) and one-way ANOVA with *a* post hoc *Tukey test* (A, E and G).

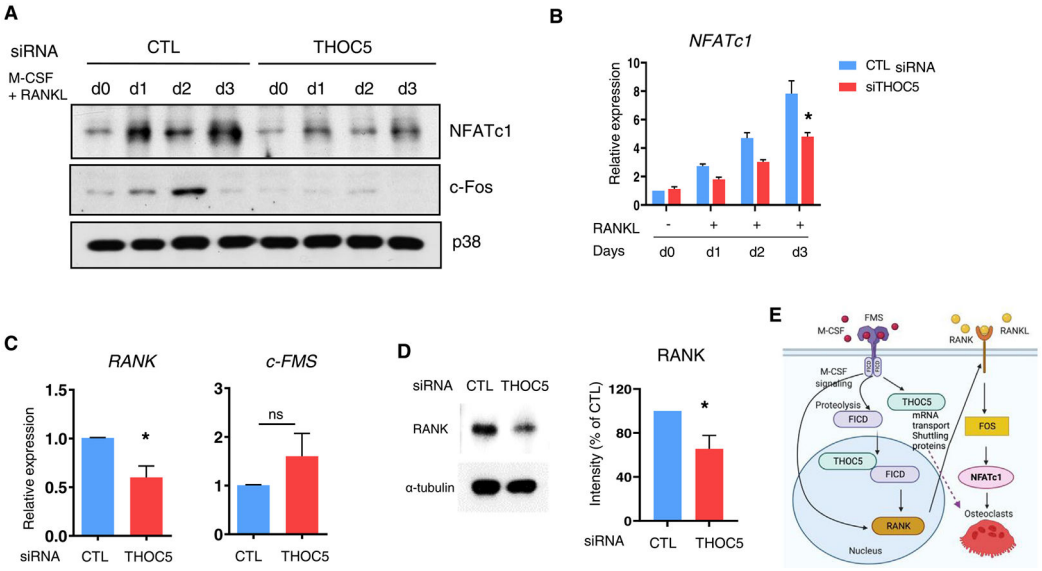

Fig. 4.

THOC5 regulates RANK expression in human OCPs. Human monocytes were nucleofected with negative control (CTL) or THOC5-specific small interfering RNAs (siRNAs). Control and THOC5 KD OCPs were cultured with M-CSF (20 ng/ml) and RANKL (40 ng/ml) for 4 days. (A) Immunoblot analysis of NFATc1 and c-Fos after stimulation of human OCPs with RANKL for the indicated times (n = 3). (B) RT-PCR analysis of *NFATc1* mRNA, normalized relative to *GAPDH* mRNA (n = 3). (C and D) CTL and THOC5 KD OCPs were cultured with M-CSF (20 ng/ml) for 24 h. (C) RT-PCR analysis of RANK and c-Fms (n = 5) (D) Immunoblot analysis of RANK expression. *Right*, Representative images. *Left*, Quantification of immunoblot intensity (% of CTL) (n = 4). (E) A schematic summarizing the role of THOC5 in human osteoclastogenesis. All data represent mean ± SEM. * , $p < 0.05$; n.s, not-significant by one-way ANOVA with *a* post hoc *Tukey test* (B) and Kolmogorov-Smirnov test (C and D).