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Downregulation of Notch Modulators, Tetraspanin 5 and 10, Inhibits Osteoclastogenesis in vitro

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Abstract

Genetic studies in human and mice have pinpointed an essential role of Notch signaling in osteoblast and osteoclast differentiation during skeletal development and bone remodeling. However, the factors and pathways regulating Notch activation in bone cells remain largely unknown. In this *in vitro* study, we have provided evidence that two of the TspanC8 subfamily members of tetraspanins, Tspan-5 and Tspan-10, are up-regulated during osteoclast differentiation and knockdown of their expression by shRNAs dramatically inhibits osteoclastogenesis. Loss of Tspan-5 and Tspan-10 in osteoclast lineage cells results in attenuation of ADAM10 maturation and Notch activation. Therefore, these two tetraspanins play a critical role in osteoclast formation, at least in part, by modulating Notch signaling pathway.

Keywords

Tetraspanin; ADAM10; Notch; Osteoclast; Bone resorption

Introduction

Bone resorption by osteoclasts and bone formation by osteoblasts are essential for skeletal development and homeostasis. In adults, bone mass is maintained by a process called bone remodeling, in which bone resorption is coupled to bone formation in such a way that the amount of bone removed by osteoclasts is precisely replaced by new bone deposition by osteoblasts [1]. Under pathological conditions, however, excessive bone resorption, due to either increased osteoclast number or enhanced activity, leads to bone loss and is responsible for metabolic bone diseases such as postmenopausal osteoporosis, rheumatoid arthritis, Paget's disease of bone, and lytic tumor bone metastasis [2]. Thus, deciphering the cellular

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and molecular mechanisms of osteoclast differentiation and function is both biologically and clinically important as it holds the promise of developing novel therapeutic targets for the treatment of metabolic bone diseases.

Osteoclasts are highly specialized polykaryons that are capable of resorbing calcified cartilage and bone matrix during skeletal growth and remodeling [3, 4]. They are formed by fusion of mononuclear precursors of the monocyte/macrophage lineage under the control of two key cytokines, receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), which regulate the proliferation and differentiation of osteoclast progenitors and the survival of mature cells. Other cell surface receptors, especially immunoglobulin-like receptors and their associated adapter proteins, potentiate osteoclast differentiation by enhancing RANKL-induced Ca^{2+} mobilization and activation of NFATc1, the master transcription factor of osteoclastogenesis [5]. Recent genetic studies in human and mice have revealed an essential role of Notch receptor signaling in bone development and homeostasis [6, 7].

The Notch signaling pathway is evolutionarily conserved and plays a critical role in the determination of cell fate during embryonic development and postnatal tissue homeostasis [8]. Both Notch receptors and ligands are expressed in osteoclast lineage cells. Deletion of Notch receptors in osteoclast precursors enhances osteoclast formation and bone resorption, suggesting an inhibitory role of Notch in osteoclastogenesis [9]. On the other hand, activation of Notch signaling, especially Notch2, promotes osteoclast differentiation [10, 11]. These seemingly contradictory findings warrant further characterization of the spatio-temporal and cell-context regulation of Notch signaling in osteoclasts.

Each Notch receptor is present at the cell surface as a heterodimer composed of N- and C-terminal fragments. When the cognitive ligand binds to the extracellular domain of Notch, a disintegrin and metalloprotease (ADAM), such as ADAM10, cleaves the juxtamembrane S2 proteolysis site of the Notch receptor [12]. The γ-secretase subsequently cleaves the S3 site within the transmembrane domain and releases the Notch intracellular domain (NICD). NICD then translocates to the nucleus, where it functions as a transcriptional co-activator of Notch target genes, including members of the Hes (hairy and enhancer of split) and Hey (Hes related with YRPW motif) families [13].

The tetraspanin family of proteins is composed of a group of 33 proteins characterized by four-transmembrane passes with unique structural features [14]. These proteins interact with one another and with other integral membrane proteins to form plasma membrane microdomains, known as tetraspanin webs or tetraspanin enriched-microdomains (TEM) [15]. Thereby, tetraspanins are involved in several cellular processes such as cell adhesion, migration, cell fusion, and signal-modulation of membrane receptors [16, 17]. More recently, the TspanC8 subgroup of tetraspanins, including Tspan-5, -10, -14, -15, -17, -33, with eight cysteine residues within their second extracellular region have been shown to promote Notch activation through regulation of ADAM 10 maturation and cell surface expression in C elegans, Drosophila, and mammalian cells [18–20]. The tetraspanin CD9 has been found to regulate cell fusion during osteoclastogenesis, though contradictory findings have been reported [21, 22]. It has been reported that Tspan-5 is up-regulated

during osteoclast differentiation and that it promotes RANKL-induced osteoclast formation [23]. However, whether Tspan-5 modulates Notch activation in osteoclast lineage cells has not been determined.

In this study, we present evidence that the expression of Tspan-5 and Tspan-10 increases during osteoclast differentiation. Down-regulation of the expression of these two genes by sh-RNA mediated gene-silencing blunts osteoclastogenesis in vitro, in part because of impaired Notch activation.

Materials and Methods

Antibodies and reagents

Antibodies were purchased from the following sources: mouse monoclonal anti-Cathepsin K (182-12G5) (Millipore); rat anti-Notch1 (bTAN 20) and rat anti-Notch2 (C651.6DbHN) (Developmental Studies Hybridoma Bank); Rabbit polyclonal anti-ADAM 10 (AB19026) (Millipore); Rabbit polyclonal anti-Hes1 (ab157181) (abcam); mouse monoclonal anti-alpha tubulin (Sigma). Alpha-MEM and Penicillin-Streptomycin-L-Glutamine (PSG) were purchased from Life Technologies and Sigma-Aldrich, respectively. Fetal bovine serum was purchased from Hyclone.

Bone marrow macrophage and osteoclast cultures

Bone marrow macrophages (BMMs) were prepared as described previously [24]. Briefly, whole bone marrow was isolated from tibiae and femora of 8–10 weeks C57BL/6 mice. Bone marrow cells were plated in α-10 medium (α-MEM, 10% heat-inactivated fetal bovine serum, 1 × PSG solution) containing 1/10 volume of CMG 14–12 (conditioned medium supernatant containing recombinant M-CSF at 1μg/ml) [25] in petri-dishes. Cells were incubated at 37°C in 5% CO_2, 95% air for 4–5 days. Fresh media and CMG 14–12 supernatant were replaced every the other day. Osteoclasts were generated after five days culture of BMMs with 1/100 volume of CMG 14–12 supernatant and 100 ng/ml of recombinant RANKL.

TRAP (tartrate-resistant acid phosphatase) staining

BMMs were cultured on 48-well tissue culture plate in α-10 medium with M-CSF and RANKL for 4–5 days. The cells were fixed with 4% paraformaldehyde/PBS and TRAP was stained with NaK Tartrate and Napthol AS-BI phosphoric acid (Sigma-Aldrich) as described previously [26].

Quantitative real time RT-PCR

BMMs were cultured in 6-well plates with M-CSF and/or RANKL for 5 days. Total RNA was purified from three independent cultures of each group using RNeasy mini kit (Qiagen) according to the manufacture's protocol. First-strand cDNAs were synthesized from 0.5–1 μg of total RNA using the High Capacity cDNA Reverse Transcription kits (Life Technologies) following the manufacturer's instructions. TaqMan quantitative real-time PCR was performed using the following assays from Life Technologies: *Tspan5* (Mm00497960_m1); *Tspan10* (Mm01264401_m1); *Acp5* (Mm00475698_m1); *Hes1*

(Mm01342805_m1); *Hey1* (Mm00468865_m1). Samples were amplified using the StepOne Plus real-time PCR system (Life Technologies) with an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative mRNA amounts was calculated by normalizing to the mitochondrial gene Mrps2 mRNA, which is steadily expressed in both BMMs and osteoclasts, using the ΔCt method [27].

Lentivirus mediated shRNA expression

The LKO.1 lentiviral vectors expressing shRNA sequences targeting the mRNAs of murine Tspan-5 (TRCN0000094994/NM_019571.1-2641s1c1 and TRCN0000094995/ NM_019571.1-862s1c1) and murine Tspan-10 (TRCN0000094620/NM_145363.1-648s1c1 and TRCN0000094621/ NM_145363.1-161s1c1) were purchased from Sigma-Aldrich. 293-T cells were co-transfected with a LKO.1 gene transfer vector and virus packaging vectors, ΔH8.2 and VSVG using TransIT-LT1 transfection reagent (Mirus). Virus supernatants were collected after 48 hours transfection. BMMs were transduced with virus supernatant containing M-CSF and 20 μg/ml of protamine (Sigma-Aldrich). Cells were then selected in α-10 medium containing M-CSF and 6 μg/ml puromycin (Sigma-Aldrich) for 3 days [28].

Immunoblotting

Cultured cells were washed with ice-cold PBS twice and lysed in 1 × RIPA buffer (Sigma) containing 1 mM DTT and complete mini EDTA-free protease inhibitor cocktail (Roche). After incubation on ice for 30 min, the cell lysates were clarified by centrifugation at 14,000 rpm for 15 min at 4 °C. Ten to thirty μg of total protein were subjected to SDS-PAGE gels and transferred electrophoretically onto PVDF membrane by a semi-dry blotting system (Bio-Rad). The membrane was blocked in 5% fat-free milk/Tris-buffered saline for 1 h and incubated with primary antibodies at 4°C overnight followed by secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology). After rinsing 3 times with Tris-buffered saline containing 0.1% Tween 20, membranes were developed with enhanced chemiluminescent detection reagents (Millipore).

Statistics

Data of 2-group comparisons were analyzed using a 2-tailed Student's *t* test. Data are represented as mean ± SD.

Results

Expression of Tspan-5 and Tspan-10 is up-regulated during osteoclastogenesis in vitro

The Notch signaling pathway has been shown to regulate osteoclast differentiation either directly on osteoclast precursors or indirectly through osteoblasts [9]. To identify modulators of Notch signaling in osteoclast lineage cells, we examined the expression of TspanC8 subfamily members of tetraspanins, which have been shown to promote ADAM10/ Notch activation in mammalian cells, in the course of osteoclast differentiation from bone marrow macrophages *in vitro*. As shown in Figure 1, the mRNA expression of Tspan-5 and Tspan-10, determined by real-time quantitative RT-PCR, increases during osteoclast differentiation, reaching their highest levels in mature cells. Of the other TspanC8 subfamily members (Tspan-14, -15, -17, and -33), Tspan-14 and Tspan-17 mRNAs were detectable but

their expression remained steady in macrophages (BMM), pre-osteoclasts (pOC), and mature osteoclasts (OC) (data not shown). These results suggest that Tspan-5 and Tspan-10 might be functionally important in osteoclast formation and/or function.

Knock-down of Tspan-5 expression in BMMs attenuates osteoclast differentiation via decreasing Notch activation

To define the role of Tspan-5 and Tspan-10 in osteoclast lineage cells, we first knocked down Tspan-5 expression in BMMs by lentiviral transduction of two shRNAs (Tsp5-sh1 and Tsp5-sh2) which specifically target different sites of murine Tspan-5 mRNA. A shRNA targeting firefly luciferase (Luc-sh) was used as a negative control. Real-time PCR analysis showed that both shRNAs significantly reduced Tspan-5 expression in BMMs (Figure 2A). Since Tsp5-sh2 was more potent than Tsp5-sh1 in inhibiting Tspan-5, we subsequently used cells expressing this shRNA to examine the effects of Tspan-5 knockdown on osteoclastogenesis. As demonstrated in Figure 2B, Tspan-5 deficiency dramatically attenuated osteoclast formation after 4 and 5 days of culture of BMMs with RANKL and M-CSF, as determined by histological staining for TRAP, an osteoclast differentiation marker. This evidence was reinforced by the decrease of RANKL-induced TRAP (encoded by *Acp5* gene) mRNA expression and Cathepsin K protein expression in Tspan-5 deficient osteoclasts, as compared to control cells (Figure 2C and 2D). In consistence with recent reports in other cells [18–20], down-regulation of Tspan-5 expression in osteoclast precursors prevented ADAM10 maturation and Notch2 activation upon RANKL stimulation (Figure 2E). In accordance with its role in regulating Notch activation in other cell types, loss of Tspan-5 in macrophages decreased the expression of two Notch target genes, *Hey1* and *Hes1*, as measured by real-time quantitative PCR (Figure 2F). Taken together, these results reveal an important function of Tspan-5 in activating Notch signaling and promoting RANKL-induced osteoclastogenesis.

Tspan-10 regulates ADAM10 maturation and Notch activation during osteoclastogenesis

Next we attempted to elucidate the function of Tspan-10, the expression of which was also found up-regulated during osteoclastogenesis. By lenti-viral transduction, two specific shRNAs greatly reduced Tspan-10 expression in macrophages and mature osteoclasts, as revealed by quantitative real-time PCR (Figure 3A). Similar to the effects of Tspan-5 down-regulation on osteoclastogenesis, loss of Tpasn-10 in osteoclast lineage cells impaired osteoclast formation, as illustrated by a decreased number of TRAP positive multi-nucleated osteoclasts in Tspan-10 knock-down cultures compared to cultured control cells (Figure 3B). This result was further confirmed by diminished TRAP and Cathepsin K expression in response to RANKL in Tspan-10 deficient osteoclast lineage cells as compared to control cells (Figure 3C and 3D).

It has been recently reported that the TspanC8 subfamily members of tetraspanins promote Notch activation via interacting with ADAM10 and regulating its maturation and trafficking to the cell surface [20], where ADAM10 cleaves the juxtamembrane S2 proteolysis site of Notch receptor. Earlier work by Verrier *et al* has shown expression and plasma membrane localization of ADAM10 in osteoclasts [29]. To determine whether Tspan-10 regulates Notch signaling through ADAM10 in osteoclast lineage cells we examined the protein level

of ADAM10 in macrophages upon RANKL stimulation. As shown in Figure 4A, the low molecular weight form of ADAM10, which corresponds to mature ADAM10 with a cleaved prodomain, was diminished in Tspan-10 deficient cells compared to controls. Consistent with the role of ADAM10 in Notch activation, the protein levels of NICD2 and Hes1 were reduced in RANKL-stimulated Tspan-10 deficient cells compared to controls (Figure 4A). In contrast to a previous report [9], we could not detect NICD1 protein in macrophages and osteoclasts by western blot using an antibody from Developmental Studies Hybridoma Bank (Figure 4B). The cell lysate from a human Merkel cell carcinoma cell line served as a positive control of the antibody. A similar result was obtained using an anti-Notch1 antibody from Cell Signaling Technologies, LLC (data not shown). This result, together with the finding by Fukushima et al [10], indicate that Notch 2 is the major Notch receptor in osteoclast lineage cells. Whether Notch1 activation was suppressed by downregulation of Tspan-5 or Tspan-10 remain unclear. Furthermore, the mRNA expression of Notch target genes, *Hes1* and *Hey1*, was markedly decreased in Tspan-10 knocking down osteoclasts, suggesting attenuation of the transcriptional activities of Notch. Collectively, the results presented in this study indicate that Tspan-5 and Tspan-10 of tetraspanins play an important role in osteoclastogenesis probably through the regulation of ADAM10 and Notch activation.

Discussion

Recent genetic studies in humans and mice have uncovered an essential role of Notch signaling in osteoblast and osteoclast differentiation during skeletal development and bone remodeling in adults [11, 30, 31]. However, factors and pathways regulating Notch activation in bone cells remain largely unknown. The sequential proteolytic cleavages mediated by ADAM10 and a γ-secretase complex, respectively, are key steps in Notch activation [12]. The TspanC8 subgroup of tetraspanins has been shown to activate Notch signaling through their regulation of ADAM10 maturation and cell surface expression, a mechanism conserved from C elegans, Drosophila, to mammalian cells [18–20]. In this study, we have shown that two of the TspanC8 subfamily members, Tspan-5 and Tspan-10, were up-regulated during osteoclast differentiation. Knockdown of their expression by shRNAs dramatically inhibited osteoclastogenesis *in vitro*. Mechanistically, loss of Tspan-5 and Tspan-10 in osteoclast lineage cells was associated with attenuated ADAM10 maturation and Notch activation. Therefore, these two tetraspanins play a critical role in osteoclast formation, at least in part, by modulating Notch pathway (Figure 5).

It should be noted that there is no evidence indicating that Notch target genes, such as Hes1 and Hey1, paly a direct role in osteoclast differentiation and function. In addition, the reported functions of each Notch ligand and individual Notch receptor isoform on osteoclastogenesis are controversial. While immobilized Notch ligand Delta-1 has been reported to inhibit osteoclast differentiation in vitro [32], Notch ligand Jagged1 derived from breast cancer cells has been shown to directly activate osteoclastogenesis in bone marrow [33]. Loss of all three Notch receptors (Notch 1, 2, and 3), or Notch 1 alone, in osteoclast precursors increases osteoclast formation, indicating that Notch signaling inhibits osteoclastogenesis [9]. On the other hand, it has been shown that Notch 2 is required for osteoclast differentiation [10, 11]. The spatio-temporal and cell-context regulations of Notch

signaling in osteoclasts under both physiological and pathological conditions require further investigation. In consistent with the finding by Fukushima et al [10], we have found that the protein level of Notch2, as detected by western blots, is much higher than that of Notch1 in osteoclast lineage cells. Thus, Notch2 is the major Notch receptor regulated by Tspan-5/10 in osteoclasts.

Iwai *et al* [23] have previously reported that the expression of Tspan-5 increases during osteoclast differentiation from a monocytes/macrophage-lineage osteoclast precursor cell line, RAW264.7. Consistent with our finding in primary cultures, inhibition of Tspan-5 expression in RAW264.7 cells by short-interfering RNAs blunts osteoclastogenesis. In the present study, we further elucidated that loss of Tspan-5 in osteoclast lineage cells attenuated Notch activation. In addition to Tspan-5 and Tspan-10, Tspan-14 and Tspan-17 of the TspanC8 subfamily are also expressed in macrophages but their levels remain the same during osteoclast differentiation (data not shown). Despite high similarities in structures and their interactions with ADAM10 among TspanC8 subfamily tetraspanins, the inhibitory effects of Tspan-5 down-regulation on osteoclastogenesis and Notch activation were not compensated by Tspan-10 or other subfamily members and vice versa, suggesting that Tspan-5 and Tspan-10 may regulate Notch activation in osteoclasts independently. Alternatively, they may form independent units or microdomains at the plasma membrane, as other tetraspanins do [15]. Loss of either of them leads to decreased cell surface level of ADAM10 and subsequent attenuation of Notch receptor processing and activation. Future work will be needed to fully characterize the molecular action of Tspan-5 and Tspan-10 on osteoclastogenesis and find whether Tspan-14 and Tspan-17 also play a role in osteoclast differentiation through regulating ADAM10 and Notch signaling. Since the mechanisms by which TspanC8 subfamily of tetraspanins activate Notch signaling are conserved from Caenorhabditis. Elegans, Drosophila, to human cells [18, 19], it will be worthy to examine the expression and functions of TspanC8 tetraspanins in human osteoclasts. Unfortunately, detection of protein expression and examination of their localization of endogenous Tspan-5 and Tspan-10 have not been done at the moment, due to lack of suitable antibodies. It has been shown that Notch stimulates proliferation in osteoblast precursors but inhibits their final differentiation from mesenchymal stem cells to mature osteoblasts [30, 31, 34]. Thus, it will be interesting to define the role of TspanC8 tetraspanins in Notch regulation in osteoblasts and determine whether they play a role in bone remodeling and homeostasis using genetically modified mouse models of TspanC8 subfalimy members.

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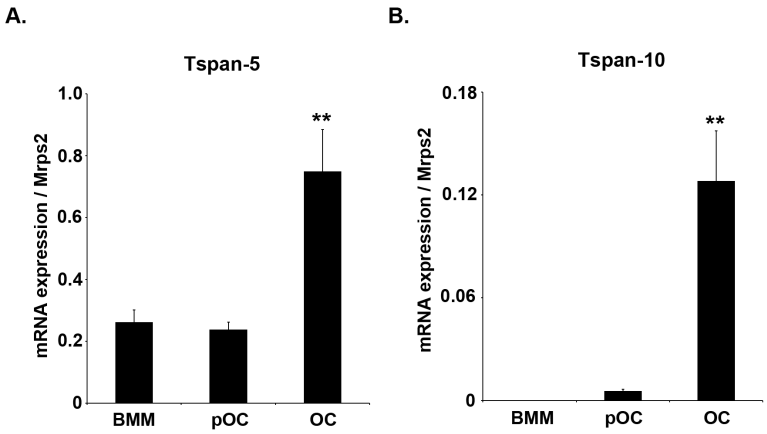

Fig. 1.

The expression of Tspan-5 and Tspan-10 is up-regulated during osteoclast differentiation. Total RNAs were purified from three independent cultures of macrophages (BMM), pre-osteoclasts (pOC), and mature osteoclasts (OC), respectively. Quantitative real-time RT-PCR analysis of the expression of Tspan-5 (A) and Tspan-10 (B) mRNA during osteoclast differentiation was performed using TaqMan assay primers from Life Technologies. ** $p < 0.01$ vs BMM by Student's t-test.

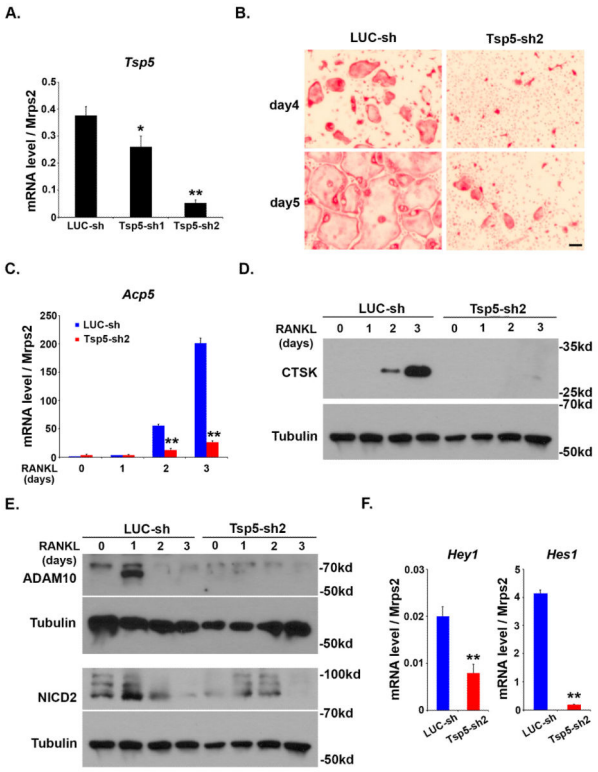

Fig. 2.

Knock-down of Tspan-5 expression markedly inhibits osteoclast formation and Notch activation. (A) BMMs were transduced with recombinant lenti-viruses expressing a control shRNA targeting fire fly luciferase (LUC-sh) or two Tspan-5-targeting shRNAs (Tsp5-sh1 and Tsp5-sh2), respectively. After selecting with 6 μg/ml puromycin for 3 days, total RNAs were isolated from three independent BMM cultures of each group. Quantitative real-time RT-PCR analysis of the expression of Tspan-5 was performed. * $p < 0.05$, ** $p < 0.01$ vs LUC-sh by Student's t-test. (B) Lenti-viruses transduced BMMs were cultured with M-CSF and RANKL for 5 days. The cells were fixed and stained for TRAP. The scale bar = 20 μm. (C) The mRNA expression of the osteoclast marker gene, TRAP (encoded by *Acp5*) was measured by quantitative real-time PCR using TaqMan assay primer from Life Technologies., n = 3, ** $p < 0.01$ vs LUC-sh by Student's t-test. (D) The protein expression of Cathepsin K was detected by western blots. Tubulin served as a loading control. (E) The protein level of ADAM10, NICD2 was detected by western blots. Tubulin served as a loading control. (F) The mRNA expression of Notch target genes, *Hes1* and *Hey1*, in BMMs was measured by quantitative real-time PCR using TaqMan assay primers from Life Technologies., n = 3, ** $p < 0.01$ vs LUC-sh by Student's t-test.

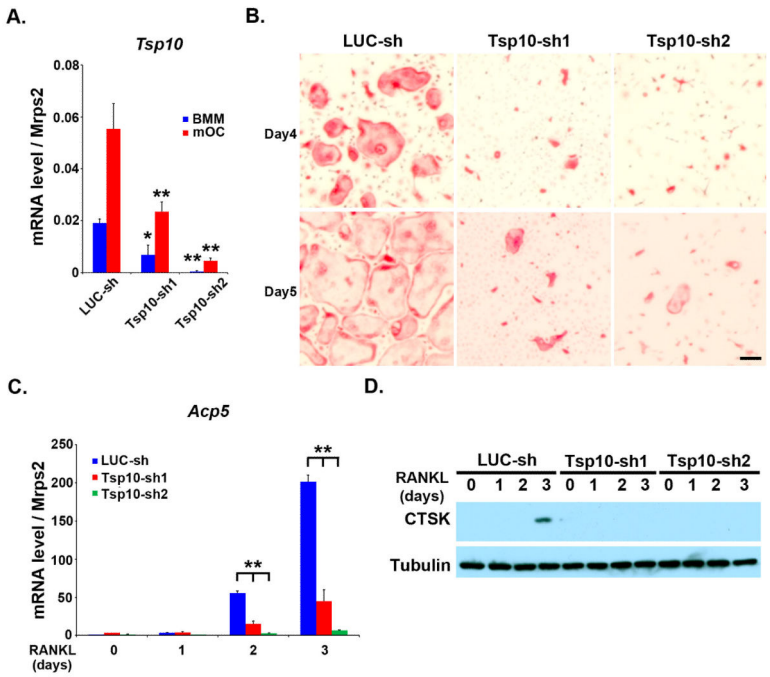

Fig. 3.

Knocking down of Tspan-10 expression attenuates osteoclast formation. (A) BMMs were transduced with recombinant lenti-viruses expressing a control shRNA targeting fire fly luciferase (LUC-sh) or two Tspan-10-targeting shRNAs (Tsp10-sh1 and Tsp10-sh2), respectively. After selecting with 6 μg/ml puromycin for 3 days, the cells were cultured with M-CSF alone (BMM) or with M-CSF plus RANKL for 5 days (mOC). Total RNAs were isolated from three independent cultures of each group. Quantitative real-time RT-PCR analysis of the expression of Tspan-10 was performed. * $p < 0.05$, ** $p < 0.01$ vs LUC-sh by Student's t-test. (B) Lenti-viruses transduced BMMs were cultured with M-CSF and RANKL for 5 days. The cells were fixed and stained for TRAP. The scale bar = 20 μm. (C) The mRNA expression of osteoclast marker gene, TRAP (encoded by *Acp5*), was measured by quantitative real-time PCR using TaqMan assay primers from Life Technologies., n = 3, ** $p < 0.01$ vs LUC-sh by Student's t-test. (D) The protein expression of Cathepsin K was detected by western blots. Tubulin served as a loading control.

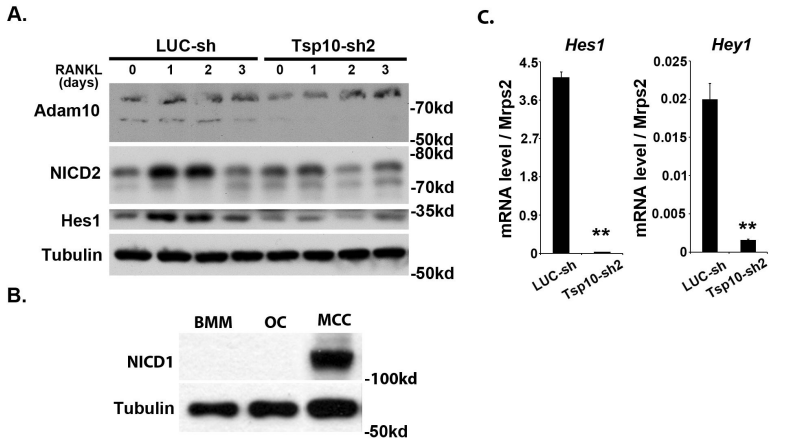

Fig. 4.

Loss of Tspan-10 in osteoclast lineage cells impairs ADAM10 maturation and Notch activation. (A) Lentiviral transduced BMMs were either cultured with M-CSF alone or M-CSF plus RANKL for the indicated time. The protein level of ADAM10, NICD2 and Hes1 was detected by western blots. Tubulin served as a loading control. (B) The protein level of Notch1 in bone marrow macrophages (BMM) and osteoclasts (OC) was detected by western blots using an antibody from Developmental Studies Hybridoma Bank. The cell lysate from a human Merkel cell carcinoma cell line served as a positive control of the antibody. Tubulin served as a loading control. (C) The mRNA expression of Notch target genes, *Hes1* and *Hey1*, in BMMs was measured by quantitative real-time PCR using TaqMan assay primers from Life Technologies., n = 3, ** $p < 0.01$ vs LUC-sh by Student's t-test.

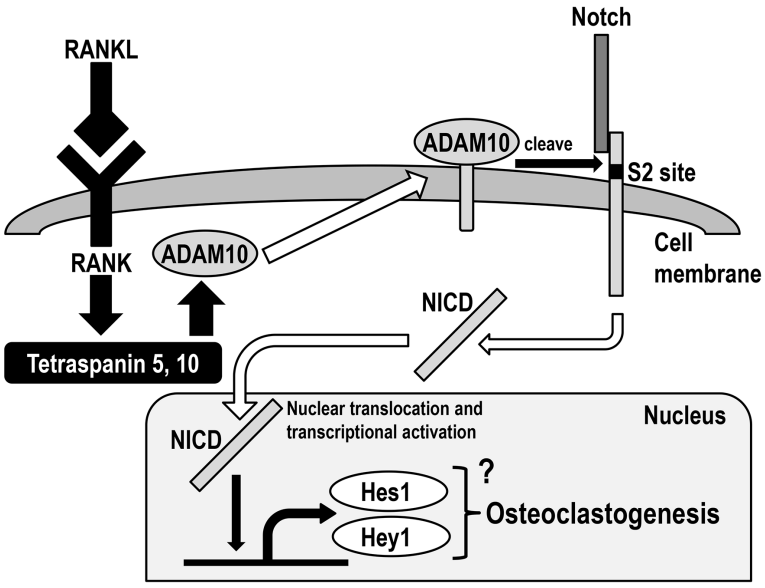

Fig. 5.

A working model of the role of Tspan-5 and -10 in Notch activation and osteoclastogenesis.