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Tunneling Nanotube Formation Is Essential for the Regulation of Osteoclastogenesis

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ABSTRACT

Osteoclasts are the multinucleated giant cells formed by cell fusion of mononuclear osteoclast precursors. Despite the finding of several membrane proteins involving DC-STAMP as regulatory proteins required for fusion among osteoclast precursors, cellular and molecular events concerning this process are still ambiguous. Here we identified Tunneling Nanotubes (TNTs), long intercellular bridges with small diameters, as the essential cellular structure for intercellular communication among osteoclast precursors in prior to cell fusion. Formation of TNTs was highly associated with osteoclastogenesis and it was accompanied with the significant induction of the M-Sec gene, an essential gene for TNT formation. M-Sec gene expression was significantly upregulated by RANKL-treatment in osteoclast precursor cell line. Blockage of TNT formation by Latrunclin B or by M-Sec siRNA significantly suppressed osteoclastogenesis. We have detected the rapid intercellular transport of not only the membrane phospholipids labeled with DiI but also the DC-STAMP-GFP fusion protein through TNTs formed among osteoclast precursors during osteoclastogenesis. Transportation of such regulatory molecules through TNTs would be essential for the process of the specific cell fusion among osteoclast precursors. J. Cell. Biochem. 114: 1238–1247, 2013.

KEY WORDS: MEMBRANE NANOTUBE; OSTEOCLAST DIFFERENTIATION; DC-STAMP; M-SEC GENE; TIME-LAPSE ANALYSIS; GFP-FUSION PROTEIN; MEMBRANE PHOSPHOLIPID

Tunneling membrane nanotubes (TNTs) are intercellular bridges bearing cylindrical structure with small diameters of nanometer level with length of 10–100 μm or more [Davis and Sowinski, 2008; Zani and Edelman, 2010; Domhan et al., 2011]. It has recently been recognized that TNTs play important roles in cell-to-cell communication among immune cells [Davis and Sowinski, 2008; Arkwright et al., 2010; Zhang, 2011]. It has been shown the rapid transportation of membrane surface molecules, Ca^{+} signals, small intracellular organelles through TNTs [Davis and Sowinski, 2008; Smith et al., 2011]. By using the intercellular network formed by TNTs, virus particles can be spread rapidly to adjacent cells to cause accelerated spreading of virus infection [Sowinski et al.,

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2010]. NK cells utilize TNTs to communicate not only with other NK cells but also with target cells [Chauveau et al., 2010; Delogu et al., 2011]. Recently, M-Sec gene has been revealed to be an essential gene for TNT formation [Hase et al., 2009]. M-Sec protein expressed in macrophage and dendritic cells induces formation of TNTs [Kondratiev et al., 2011].

Osteoclasts, the only bone resorbing cells, are multinucleated giant cells formed by cell fusion of mononuclear osteoclast precursors derived from hematopoietic stem cells [Roodman et al., 1985; Teitelbaum, 2007]. Osteoclast differentiation is mainly induced by the receptor activator NFκB ligand (RANKL) [Yasuda et al., 1998; Suda et al., 1999]. In spite of a marked progress in the study of intracellular signaling mechanism downstream of RANK (receptor for RANKL), molecular events concerning the fusion among osteoclast precursors remain to be ambiguous.

Previously, we have identified DC-STAMP, a transmembrane protein having seven transmembrane domain as a molecule essential for osteoclastogenesis [Kukita et al., 2004]. Yagi et al. [2005] has shown that DC-STAMP governs the fusion process among osteoclast precursors as well as fusion of macrophages. Several other molecules are also considered to have regulatory roles in fusion of osteoclast precursors; for example macrophage fusion receptors, CD47 [Vignery, 2000, 2005] and CD44 [de Vries et al., 2005; Cui et al., 2006]. To understand the molecular mechanism of the fusion process, it could be essential to clarify the details in the active cellular events concerning the communication among osteoclast precursors in conjunction with the dynamic behavior of these molecules regulating fusion process. This study focused on elucidating an involvement of TNTs in the fusion process of osteoclast precursors. We also estimated a possible transportation of DC-STAMP protein among osteoclast precursors through TNTs.

MATERIALS AND METHODS

ANIMALS AND CELL CULTURE

Rat osteoclasts were differentiated from bone marrow cells obtained from femur and tibia of SDS rats as described previously [Kukita et al., 1993ab]. To confirm formation of rat osteoclasts, cells were stained for osteoclast-specific monoclonal antibody Kat1 (IgM) as described previously [Kukita et al., 1994, 1998, 2001]. Murine osteoclasts were differentiated from bone marrow macrophages obtained from femur and tibia of C57BL/6J mice as described by Takahashi et al. [2003]. Murine osteoclasts were also differentiated from osteoclast precursor cell line RAW-D cells as described previously [Kukita et al., 2004; Watanabe et al., 2004]. Briefly, RAW-D cells were cultured in α-MEM containing 10% FBS for 0–3 days in the presence of 20 ng/ml RANKL and 1 ng/ml TNF-α. All experiments using animals were performed according to the "Care and Use of Animals in Kyushu University."

PLASMIDS AND TRANSFECTION

For transient expression of DC-STAMP, RAW-D cells (3×10^6 cells) were transfected with DC-STAMP cDNA inserted into pEGFP-N1 expression vector [Kukita et al., 2004] and empty vector pEGFP-N1 (Clontech Labo.) by using Nucleofector electroporation instrument (Lonza), according to the manufacturer's protocol.

siRNAS PREPARATION AND TRANSFECTION

Small interfering RNAs (siRNAs) were prepared using Silencer siRNA construction kit (Ambion). The sequences of oligonucleotides synthesized for templates are as follows; M-Sec sense template (5′-TCTCAGCCTTAGTCTGGCCTTCCTGTCTC-3′), M-Sec anti sense template (5′-GGCCAGACTAAGGCTGAGATTCCTGTCTC-3′) and mutant sense template (5′-TCTCAGCCCAAGTCTGGCCTTCCTGTCTC-3′), mutant anti sense template (5′-GGCCAGACTTGGGCTGAGATTCCTGTCTC-3′), in which two nucleotides were changed, respectively.

RAW-D cells were plated in 60 mm dish (2.5×10^4 cells per dish) or 96-well plates (1.7×10^3 cells per well) before 13.5 h of transfection. The cells were transfected with siRNAs using siPORT NEOFX transfection reagent (Ambion). After 8 h of transfection, the cells were cultured for forming osteoclasts.

MTT ASSAY

MTT assay was performed according to the manufactuer's protocol (Sigma–Aldrich Corporation).

WESTERN BLOTTING

Cell lysates were prepared as described previously [Li et al., 2009]. Equal amounts of lysate protein were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. After blocking with 5% skim milk, the membranes were probed with anti-M-Sec antibody followed by incubation with the peroxidase-conjugated anti-rabbit IgG second antibody. Antigen–antibody complexes were visualized by ECL (Amersham). The same membranes were stripped and re-probed with anti-GAPDH antibody.

IMMUNOFLUORESCENCE STAINING

Cells were fixed in 4% paraformaldehyde for 20 min at room temperature, followed by permeabilization with Triton X-100 for 4 min at room temperature. After blocking in 3% normal goat serum in PBS for 60 min at room temperature, cells were incubated with anti-mouse β-tubulin antibody, rabbit polyclonal (Santa Cruz) for 2 h. After washing, cells were incubated with goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Invitrogen). The cells were examined with Nikon A1Rsi confocal laser microscope (Nikon). Number of actin (or tubulin)-expressing TNT-like structures in the unit area (0.034 mm^2) was counted for 30 randomly selected observation fields. Percentage of actin (or tubulin)-positive cells was calculated.

RT-PCR AND REAL-TIME PCR

Total RNA was extracted using Isogen (Nippon gene). cDNA was generated using oligo-dT primers and was subjected to PCR using a RT-PCR kit (Takara). Primers for PCR reactions were listed as follows: M-Sec forward (5′-GTGCAGAACCTCTACCCCAATG-3′), M-Sec reverse (5′-TGGAGAATGTCGATGGCCCA-3′), DC-STAMP forward (5′-AAAACCCTTGGGCTGTTCTT-3′), DC-STAMP reverse (5′-CTTCGCATGCAGGTATTCAA-3′), cathepsin K forward (5′-GAGGGCCAACTCAAGAAGAA-3′), cathepsin K reverse (5′-GCCGTGGCGTTATACATACA-3′), TRAP forward (5′-CAGCTGTCCTGGCTCAAAA-3′), TRAP reverse (5′-ACATAGCCCACACCGTTCTC-3′), GAPDH forward (5′-AAACCCATCACCATCTTCCA-3′), GAPDH reverse (5′-GTGGTTCACACCCATCACAA-3′).

For Quantitative RT-PCR, total RNA was reverse transcribed with a PrimeScript RT-PCR kit (Takara) by use of random primers. Real-time PCR reactions were performed by using Taqman gene expression assay kit with a StepOnePlus real-time PCR system (Applied Biosystems). mRNA levels were normalized to Gapdh expression. Commercially available probe-primer sets (Applied Biosystem) with proprietary sequences were used.

ANTIBODIES AND CYTOKINES

Antibodies against TNF-αIP2 (=M-Sec) (rabbit IgG) and β-tubulin (rabbit IgG) were purchased from Santa Cruz Biotechnology. Goat anti-rabbit IgG antibody labeled with Alexa Flour 488 was obtained from invitrogen. Human Macrophage Inflammatory Protein-1α (MIP-1α) was purchased from Invitrogen. Human M-CSF, soluble RANK ligand, and IL-1β were obtained from Wako Pure Chemical. Human IL-6 and TNF-α were from PeproTech. Phalloidin Tetramethylrhodamine B was obtained from Sigma.

LIVE-CELL IMAGING

To investigate the transport of lipid vesicles, RAW-D cells were stained with DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate: Molecular Probes). This reagent was added 5 μl per 1 ml cell suspension and cultured for 15 min. Then cells were washed 3× with α-MEM containing 10%FBS. RAW-D cells were plated onto 35 mm non-coated glass-bottom dishes (MatTek Corp.). RAW-D cells transfected with pEGFP-N1-DC-STAMP or empty vector and those labeled with DiI were co-cultured. Cultures were observed using the deconvolution microscopy BZ-8100 (Keyence). Time-lapse images were obtained using an A1Rsi confocal laser-scanning microscope (Nikon). For observing the transportation of membrane surface molecules (or structures) and DC-STAMP proteins through TNTs, time-lapse analysis (1 min-interval) was performed for 3–8 h after 40 h from transfection.

SCANNING ELECTRON MICROSCOPIC OBSERVATION

The RAW-D cells cultured for 2 days were utilized for SEM analysis. The sample were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 30 min at room temperature followed by 1% OsO_4 in 0.1 M sodium cacodylate buffer for 1 h at 4°C. They were then dehydrated in a graded ethanol series, immersed in *t*-butyl alcohol, freeze dried with freeze-drying equipment (JFD-300, JEOL), sputter-coated with platinum, and examined under a scanning electron microscope (JSM-5400, JEOL).

STATISTICAL ANALYSIS

Each data point represents mean ± SEM from quadruplicate cultures. Statistical significance was determined using Student's *t*-test or post-analysis of variance (ANOVA) test.

RESULTS

TNTS ARE INVOLVED IN OSTEOCLASTOGENESIS

In the murine bone marrow culture system for forming osteoclasts, TNT-like intercellular bridges were observed between newly formed TRAP-positive osteoclasts (Fig. 1A left panel). TNT-like structures observed in rat osteoclastogenesis have Kat1 antigen, osteoclast specific antigen, on its surface (Fig. 1A middle and right panel). TNT-like structures were also observed in culture of osteoclast precursor cell line RAW-D cells. Although, it requires 3 days to develop maximum number of osteoclasts, TNT-like intercellular bridges were frequently observed at maximal level at 2 days of culture (Figs. 1B–D and 2A upper left panel). When osteoclastogenesis reached to the maximal level at 3 days, number of the TNT-like structures was markedly decreased (Fig. 2A lower left panel: control and Supplementary Fig. S1). Actin filaments, the essential component of TNTs [Gurke et al., 2008], were detected in TNT-like structures observed in culture of RAW-D cells at 2 days of culture (98.3 ± 0.96% of total TNT-like structure, n = 30) (Fig. 1B). Similarly, we confirmed these TNT-like structures also contain β-tubulin, the cytoskeletal protein forming microtubules (98 ± 1.15% of TNT-like structure, n = 25) (Fig. 1C). Scanning electron microscopic observation clearly showed that most of the osteoclast precursor cells possessed TNT-like structures with diameters 650–950 nm (n = 11) when cells were stimulated with RANKL and TNF-α (Fig. 1D). TNT-like structures have entirely continuous plasma membrane without junction, strongly suggesting these structures are "open ended" type, a characteristics of the typical TNTs. To identify whether formation of TNT-like structures is involved in the regulation of osteoclastogenesis, RAW-D cells were treated with Latrunclin B, an inhibitor of actin polymerization. Latrunclin B inhibited formation of TNT-like structures in a dose-dependent manner (Fig. 2A upper and middle panels). These data suggest that TNT-like structures observed in osteoclastogenesis in vitro are typical TNTs, membrane nanotubes. Latrunclin B dramatically inhibited formation of osteoclast-like multinucleated cells (MNC) in a dose-dependent manner (Fig. 2A,B). Although Latrunclin B slightly suppressed cell proliferation in these cultures (Fig. 2B right panel), its inhibition was only approximately by 10% even at higher concentrations of Latrunclin B. As shown in Figure 2C, RANKL markedly stimulated formation of TNTs (more than sixfolds) and Latrunclin-B-treatment significantly suppressed formation of TNTs.

M-SEC GENE EXPRESSION IS ESSENTIAL FOR TNT FORMATION AND OSTEOCLASTOGENESIS

To investigate whether the expression of M-Sec gene is involved in osteoclastogenesis, we examined the expression of M-Sec mRNA in osteoclastogenesis from RAW-D cells. As M-Sec gene expression is known to be induced by LPS, in prior to address the expression of M-Sec mRNA in osteoclast formation, we examined as if LPS induces M-Sec mRNA expression in RAW-D cells. As shown in Supplementary Figure S3, LPS augmented expression of M-Sec mRNA up to 1.8-folds. Expression of TRAP, DC-STAMP, and calcitonin receptor, molecular markers identifying osteoclastogenesis, was clearly induced and up-regulated in osteoclast differentiation as shown in Figure 3A and Supplementary Figure S2A. M-Sec gene expression was detected even before the onset of osteoclastogenesis, however, it was markedly up-regulated (by approximately threefolds) on Day 2 and Day 3 of culture in the presence of osteoclastogenic factors, RANKL and TNFα (Fig. 3A,B and Supplementary Fig. S2A). We also detected M-Sec gene expression in osteoclastogenesis in the system of primary bone marrow macrophages (BMMs) (Fig. 3C,D and Supplementary Fig. S2B). In this system, during the stage of BMMs

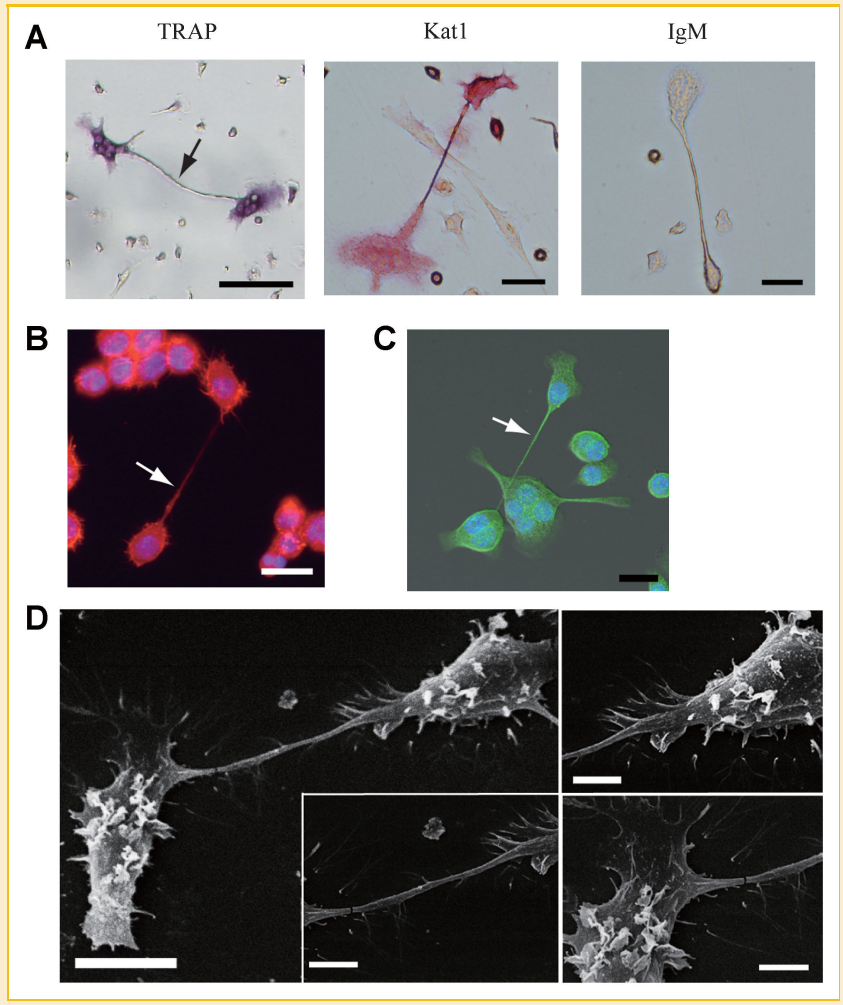

Fig. 1. TNT-like structures associated with osteoclastogenesis. A: TNT-like structure observed between newly formed between TRAP-positive osteoclasts differentiated from rat bone marrow cells. Left panel: TNT-like intercellular bridge (arrow) formed between newly formed osteoclasts from bone marrow cells on 3 days of culture (Bar: 100 μm). Center and right panels: Detection of osteoclast-specific antigen on TNT. The above cultures were stained with monoclonal antibody Kat1 (center panel) (Bar:50 μm) or control IgM (right panel) (Bar:50 μm). B,C: Expression of cytoskeletal components in TNT-like structures observed in osteoclastogenesis from RAW-D cells. RAW-D cells were cultured with RANKL and TNF-α for 2 days, then they were fixed and stained with Phalloidin-TRITC (red) (B: Bar 20 μm) or anti-β-tubulin antibody and secondary antibody labeled with Alexa Four 488 (green) (C: Bar 20 μm). D: Scanning electron microscopic observation of TNT-like structures in osteoclastogenesis. RAW-D cells were cultured with RANKL and TNF-α for 2 days followed by analysis with scanning electron microscopy. Bar: 50 μm. Right three panels: High magnification views of the left panel. Bars: 20 μm.

cultured in the presence of only M-CSF, stimulation of M-Sec gene expression was observed and maximal stimulation was seen at 1 day after RANKL-treatment. As shown in Figure 3E, numerous TNTs were observed at 1 day after RANKL-stimulation.

To investigate the role of M-Sec in osteoclastogenesis and TNT formation in osteoclast precursors, we performed knock-down experiments using siRNAs specific to M-Sec. A mutant siRNA was also prepared and utilized for control of knock-down experiments. Expression of M-Sec protein was significantly suppressed by treatment with siRNA specific to M-Sec (Fig. 4A). Osteoclastogenesis was significantly inhibited by Day 3 of the culture in RAW-D cells by knockdown of M-Sec gene expression using siRNA specific to M-Sec (Fig. 4B). Significant inhibition of TNT formation was apparent by knock-down of M-Sec expression as observed in Day 2 of culture (Fig. 4C,D). These results strongly suggest that M-Sec protein plays an important role in TNT formation in RAW-D cells and M-Sec-induced TNT formation is involved in the regulation of osteoclastogenesis. We also investigated whether other inflammatory cytokines and chemokines affect expression of M-Sec (Supplementary Fig. S3). IL1β, IL6, TNFα, and MIP-1α showed the only slight increase in the expression of M-Sec gene (1.2–1.5-folds) by the analysis with real-time PCR (Supplementary Fig. S3). In conjunction with a slight increase in M-Sec gene expression, induction of nanotube formation by inflammatory factors was observed (Supplementary Fig. S1).

MEMBRANE COMPONENTS ARE TRANSPORTED BETWEEN OSTEOCLAST PRECURSORS THROUGH TNTS IN OSTEOCLASTOGENESIS

It has recently recognized that TNTs have potent ability to transport cell surface molecules as well as virus particles [Rustom et al., 2004; Davis and Sowinski, 2008]. We next examined a possible

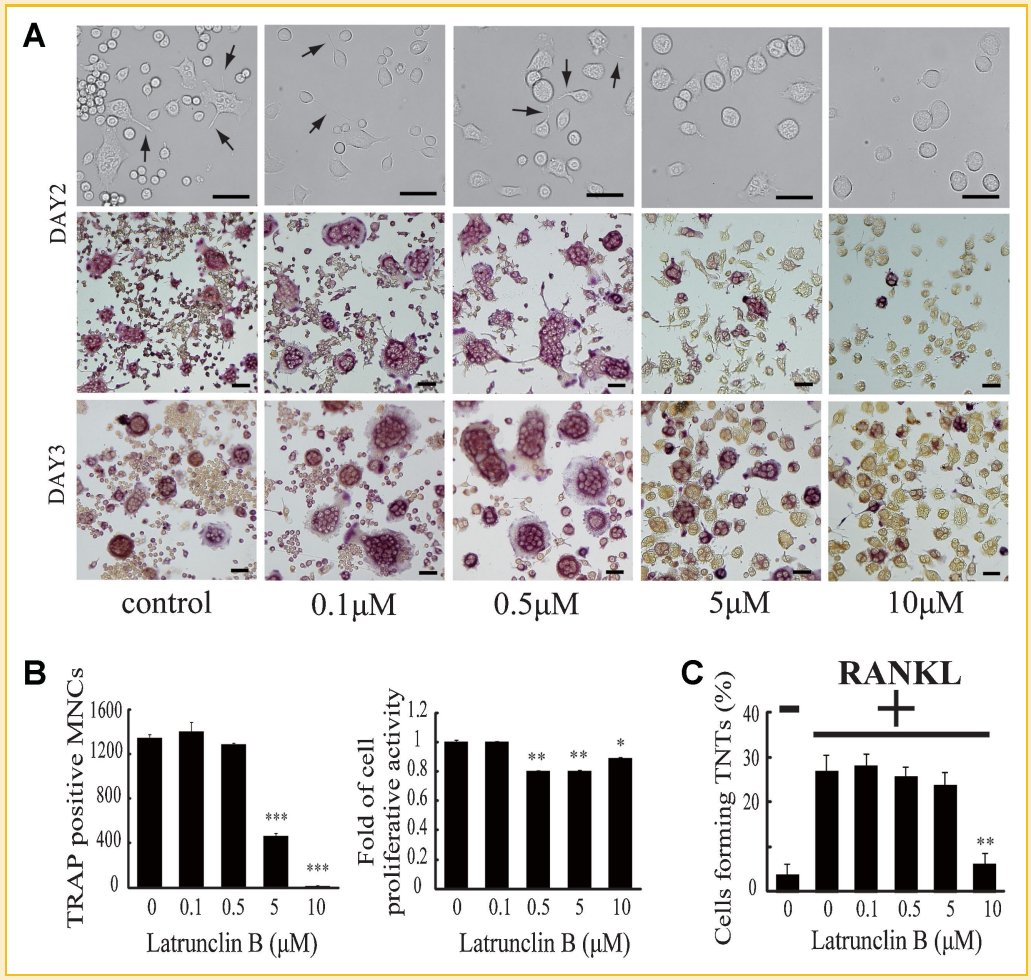

Fig. 2. Inhibition of TNT-like structure formation and osteoclastogenesis by Latrunclin B. RAW-D cells were cultured for forming osteoclasts in the presence of Latrunclin B followed by TRAP staining. A: Upper panels—Phase contrast observations. Arrows show TNT-like structures formed in culture, (Middle panels) TRAP stained cells at 2 days of culture, (Lower panels) TRAP stained cultures at 3 days of culture. Bars: 50 μm. B: Left panel—TRAP-positive osteoclast-like MNCs were counted. Data show mean number of osteoclasts ± SD from quadruplicate cultures (B, left panel). MTT assay was also performed as described in Materials and Methods Section ($n = 4$) (B, right panel). Data represent a typical experiment from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control culture in the absence of Latrunclin B. C: Induction of TNT formation by RANKL and the effect of Latrunclin B. Two days after stimulation with or without RANKL (RANKL+ or −) in the presence of various concentrations of Latrunclin B, the percentage of osteoclast precursors bearing TNTs was estimated. Quadruplicate cultures were performed and 2,000 cells per each culture were analyzed for TNT formation. Data show mean percent of TNT-bearing cells ± SD.

transportation of cell surface molecules by TNTs formed among osteoclast precursors. We co-cultured RAW-D cells transfected with GFP (green fluorescence protein) expression vector with DiI (red fluorescence dye)-labeled RAW-D cells. At 2 days of culture in the presence of RANKL and TNF-α, we performed time-lapse observation of these cultures under confocal laser microscope. We observed osteoclast precursors with high motility were actively extended and shortened TNTs toward other osteoclast precursors. Furthermore, we observed that the DiI-labeled structure involving membrane phospholipids was quickly transported from one osteoclast precursor to the other osteoclast precursor through the TNT (Fig. 5 and Supplemental video 1 for Fig. 5). These observations strongly suggest that membrane nanotubes have the ability to transport membrane phospholipids labeled with DiI. From these findings, it is considered that not only membrane phospholipids but also cell surface proteins could be transported through TNTs.

To further explore the entity of transportation of membrane molecules among osteoclast precursors through TNTs, we investigated on the transportation of DC-STAMP molecules through TNTs formed among osteoclast precursors. DC-STAMP is known to have crucial role in fusion of mononuclear osteoclast precursors [Kukita et al., 2004; Yagi et al., 2005]. We transfected RAW-D cells with the expression vector synthesizing DC-STAMP-GFP fusion protein followed by culture for forming osteoclasts. We have detected DC-STAMP-GFP protein in TNTs formed between osteoclast precursors in culture of these RAW-D cells transfected with the expression vector (Fig. 6A). We performed time-lapse observation by using confocal laser microscope. We successfully observed transportation

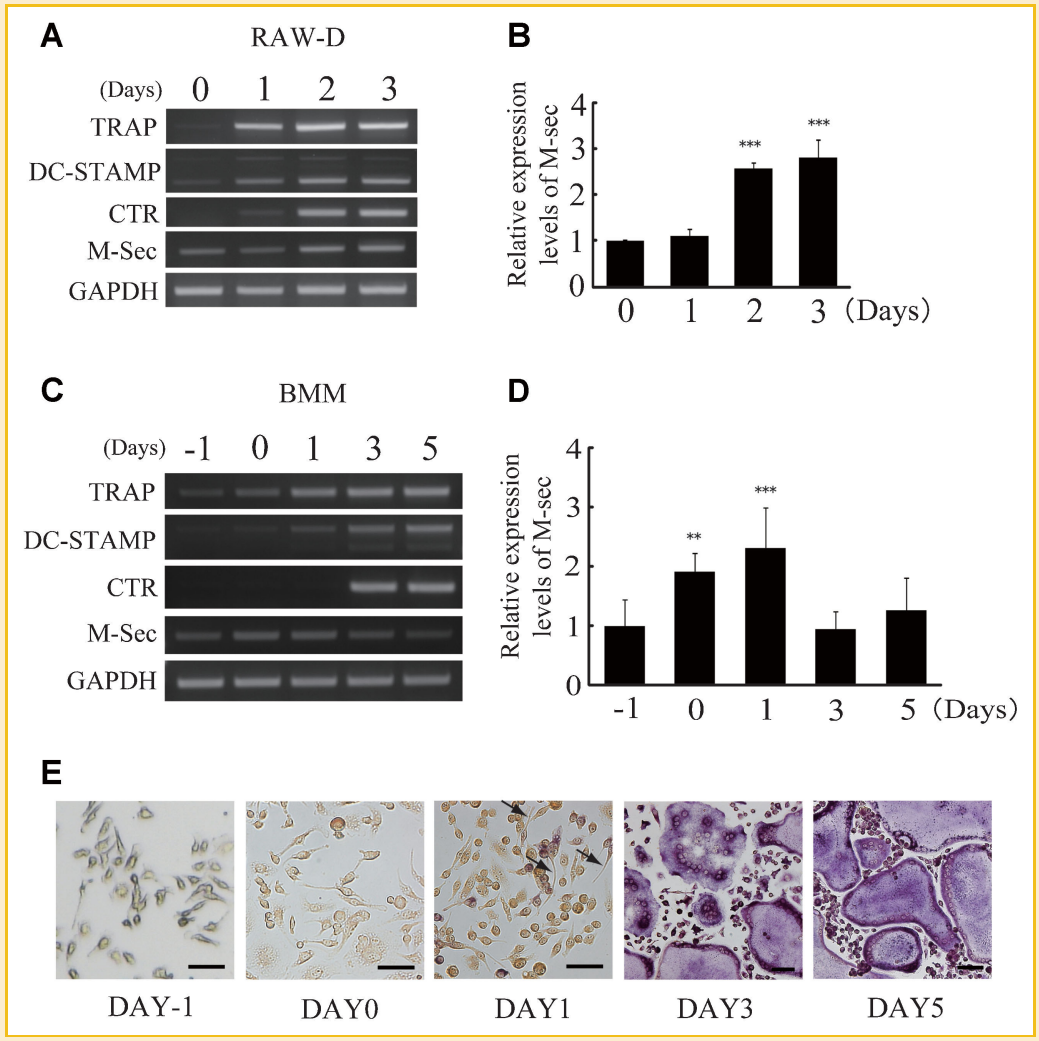

Fig. 3. Significant up-regulation of M-Sec gene expression in osteoclastogenesis. RAW-D cells were cultured by 3 days in the presence of RANKL and TNF-α. Mouse BMMs were also cultured by 5 days in the presence of RANKL and M-CSF. M-Sec gene expression was evaluated on each day of culture by use of semi-quantitative RT-PCR (A,C) or real-time PCR (B,D). $^{***}P < 0.001$ compared with the value of Day 0 (in B) or Day −1 (in D). Expression of osteoclast marker genes (TRAP, calcitonin receptor:CTR, DC-STAMP) was also analyzed (A,C). Data represent a typical experiment from three independent experiments. E: TNT formation in osteoclastogenesis from BMMs. Mouse BMM cultures was stained for TRAP on each day after stimulation with RANKL. Arrows show the typical TNTs. Bars: 50 μm.

of DC-STAMP-GFP fusion proteins through TNTs (Fig. 6B). Interestingly, the direction of DC-STAMP migration was not one way. DC-STAMP-GFP migrated back and forth along TNTs (Supplemental video 2 for Fig. 6 upper panels and Supplemental video 3 for Fig. 6 lower panels). Next, we prepared RAW-D cells transfected only with DC-STAMP-GFP expression vector and those labeled only with DiI, and equal number of these cells were mixed and cultured for forming osteoclasts, followed by observation by fluorescence microscope. In Figure 6C green cells are cells expressing DC-STAMP-GFP fusion protein and the red cells are cells stained with DiI. TNTs were observed among these cells. We found that the DiI-stained cells also have DC-STAMP-GFP fusion protein (Fig. 6C white arrows). These observations strongly suggest that DC-STAMP-GFP fusion protein expressed in osteoclast precursors have migrated into other osteoclast precursors derived from DiI-labeled RAW-D cells through TNTs. These data demonstrated a possible transportation of DC-STAMP protein through TNTs during osteoclast differentiation.

DISCUSSION

We have shown in the current paper that osteoclast precursors form numerous TNTs in osteoclastogenesis. These TNTs contain actin filaments as well as microtubules and formation of TNTs is markedly induced by RANKL in RAW-D cells. TNTs formed between osteoclast precursors had rather wide diameter of 650–950 nm probably because they have both cytoskeletal components, actin filaments, and microtubules. Inhibition of TNT-formation by Latrunclin B was highly associated with marked suppression of osteoclastogenesis by Latrunclin B. M-Sec gene expression was markedly upregulated by RANKL-treatment in RAW-D cell cultures by threefolds. Although it

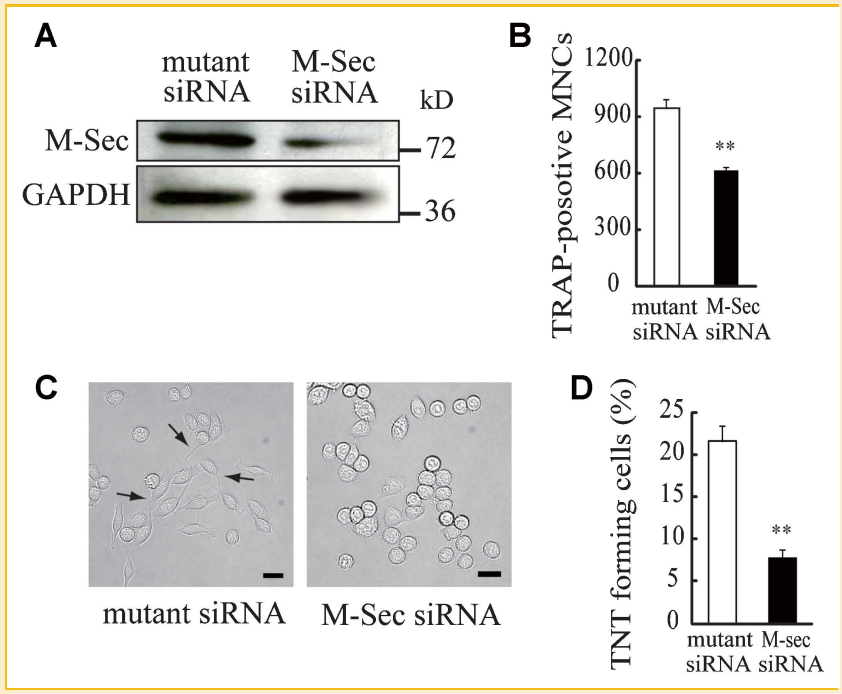

Fig. 4. Significant inhibition of TNT formation and osteoclast formation by knock-down of M-Sec gene expression. A: Knock-down of M-Sec protein expression by M-Sec siRNA in RAW-D cells. M-Sec specific siRNA (20 nM) or mutant siRNA (20 nM) were transfected to RAW-D cells followed by Western blotting analysis using anti-M-Sec and anti-GAPDH antibodies. B: Suppression of osteoclast formation by knock-down of M-Sec expression. RAW-D cells were transfected with M-Sec specific siRNA (20 nM) for 8 h, then they were stimulated with RANKL and TNF-α to form osteoclasts. Number of osteoclast-like MNCs was counted on 3 days of culture. Data show the mean number of osteoclasts $\pm$ SD from quadruplicate cultures. Data represent a typical experiment from three independent experiments. $^{**}P < 0.01$ compared with mutant siRNA control. C, D: Marked inhibition of TNT formation by siRNA specific to M-Sec in culture of osteoclastogenesis, (C) phase contrasts micrographs. Arrows show TNTs formed among osteoclast precursors. Bars: 20 μm. D: Quantitative analysis of TNT formation in RAW-D cells transfected with mutant siRNA or M-sec specific siRNA. Two days after transfection of siRNAs, the percentage of osteoclast precursors bearing TNTs was estimated. Quadruplicate cultures were performed and 2,000 cells per each culture group were analyzed for TNT formation. Data show mean percent of TNT bearing cells $\pm$ SD from quadruplicate cultures. $^{**}P < 0.01$.

was increased by LPS by 1.8-folds, only a slight increase in the M-Sec gene expression (1.2–1.5-folds) was observed by the other inflammatory factors. Upregulation of M-Sec expression was also associated with osteoclastogenesis in BMM cultures. In this system, peak of M-Sec gene expression was almost corresponded to that of TNT-formation. In RAW-D cell culture system, M-Sec specific siRNA significantly suppressed osteoclastogenesis and formation of TNTs. These lines of evidence strongly suggest the importance of TNT-formation induced by M-Sec expression in osteoclastogenesis. Interaction or exchange of regulatory molecules through TNTs among osteoclast precursors would be important in osteoclastogenesis. As the structure of TNTs is quite transient and osteoclast precursors frequently repeat formation and destruction of TNTs, in some cases, TNTs could allow cells just to communicate each other to search real partner for cell fusion in osteoclastogenesis. In RAW-D cell culture system, as the peak of TNT formation was Day 2 of culture before the peak of the osteoclastogenesis at day 3, TNTs are considered to be fully active in the process of fusion among osteoclast precursors, which is the most frequently observed at 2 days of culture. Molecular interaction via TNTs could be important to maintain efficient fusion among osteoclast precursors.

Migration of membrane components through TNTs might trigger osteoclast fusion, however, molecules transporting via TNTs had not so far been identified in osteoclastogenesis. In the current study, we have firstly shown transportation of DC-STAMP-GFP fusion protein through TNTs formed among osteoclast precursors. Although GFP fusion protein could exhibit a little different behavior in comparison to the intact DC-STAMP, our data demonstrate the strong evidence suggesting that the cell surface molecules expressed in osteoclast precursors are able to migrate and penetrate to the other precursors through TNTs. As the actual migration of cell surface molecules was detected through TNTs, the TNT type observed in our study is supposed to be the "open ended" TNT. It is strongly suggested that these TNTs are "open ended" type also from the continuous uniform distribution of cell surface Kat1 antigen [Kukita et al., 1994, 1998, 2001] on TNTs in preosteoclasts in rat osteoclastogenesis. Scanning electron microscopic observation of TNTs formed between osteoclast precursors confirmed that TNTs observed in osteoclastogenesis were "open ended" TNTs.

DC-STAMP is considered to be an essential membrane molecule for fusion among osteoclast precursors [Vignery, 2005; Yagi et al., 2005], however, the actual role of DC-STAMP in fusion process is still ambiguous. In our previous study, induction of DC-STAMP gene expression is much earlier than that of other molecular markers in osteoclastogenesis [Kukita et al., 2004], suggesting the regulatory role of DC-STAMP in the early stage of osteoclastogenesis. Transportation of DC-STAMP through TNTs may have some role in the communication among osteoclast precursors not only in the

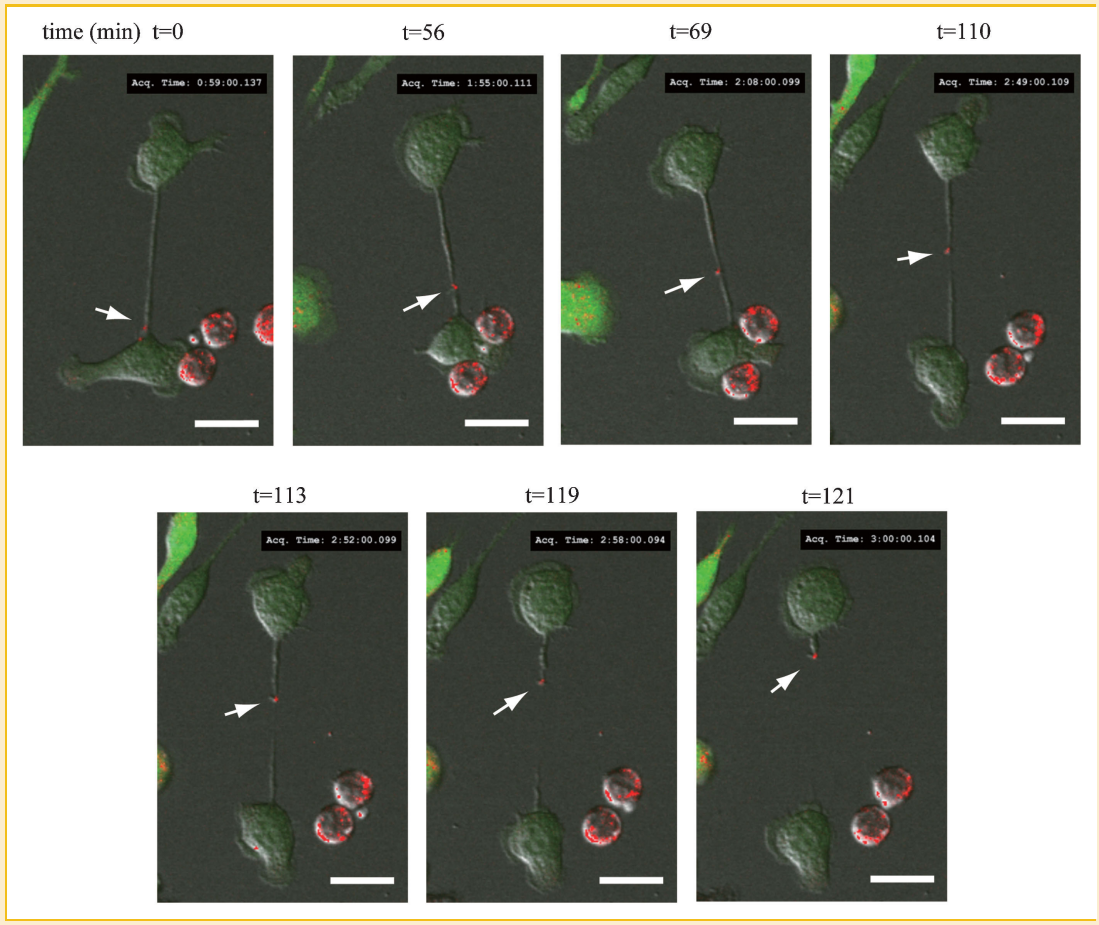

Fig. 5. Transportation of membrane phospholipids through TNTs formed among osteoclast precursors. RAW-D cells transfected with GFP expression vector (green) and those labeled with DiI (red) were mixed and cultured for forming osteoclasts. After 48 h, the time-lapse analysis (every 1 min) was performed by use of confocal laser microscope A1Rsi. Arrows: DiI-labeled membrane phospholipids migrating through TNT. Bars:20 μm.

fusion process but also in the earlier stage of osteoclastogenesis. Although, the ligand for DC-STAMP has not been identified, DC-STAMP could interact with its putative ligand expressed on the tip of TNTs or on the surface of osteoclast precursors to which DC-STAMP was penetrated through TNTs. In the current study, we have demonstrated a rapid movement of DC-STAMP-GFP protein through TNTs. It was suggested that direction of molecular movement was not unidirectional and DC-STAMP-GFP proteins rapidly move back and forth through TNTs. Such back and forth movement is likely to have proper rhythm and it may have some role to communicate among osteoclast precursors. It would be important to assess molecular entity of such rhythmical movement of DC-STAMP. Carboxyl terminus region of DC-STAMP have a motif, which permits interaction of this molecule with ERM (ezrin, radaxin, and moesin) family [Hartgers et al., 2000]. As ERM proteins are known as the membrane cytoskeleton linkers, these proteins may be involved in the interaction of DC-STAMP with cytoskeletal actin filaments. Transient and rapid movement of the cytoskeletal components would make cell surface molecules behave associate with cytoskeletal components. It should be essential to elucidate molecular events concerning migration of DC-STAMP proteins through TNTs. Jancen et al. [2009] has shown that c-terminal region of DC-STAMP is associated with OS9, amplification in osteosarcoma 9, required for Endoplasmic reticulum-to-Golgi transport and transcription factor turnover. Thus, c-terminal region of DC-STAMP could provide a regulatory domain, which could be able to determine the behavior of this molecule in association with the cytoskeletal components. Further studies are required in respect to the interaction of DC-STAMP c-terminal region with the cytoskeletal components to understand the molecular basis of the movement of DC-STAMP through TNTs.

Macrophage fusion receptor and its ligand CD47 are supposed to be involved in the fusion of macrophages as well as osteoclast precursors [Vignery, 2005]. We and other research groups have previously reported on the interaction of osteoclast precursors through binding of LFA-1 to its ligand ICAM-1 during osteoclastogenesis [Harada et al., 1998; Okada et al., 2002; Garcia-Palacios et al., 2007]. TNTs could be involved in the interaction of LFA-1 with ICAM-1 in osteoclast precursors. Investigation for the dynamic molecular behavior of such receptor-ligand systems through TNTs could provide important basic information to enlighten the molecular mechanism of fusion among osteoclast precursors. Cell-to-cell communication through TNTs might play crucial roles in osteoclastogenesis.

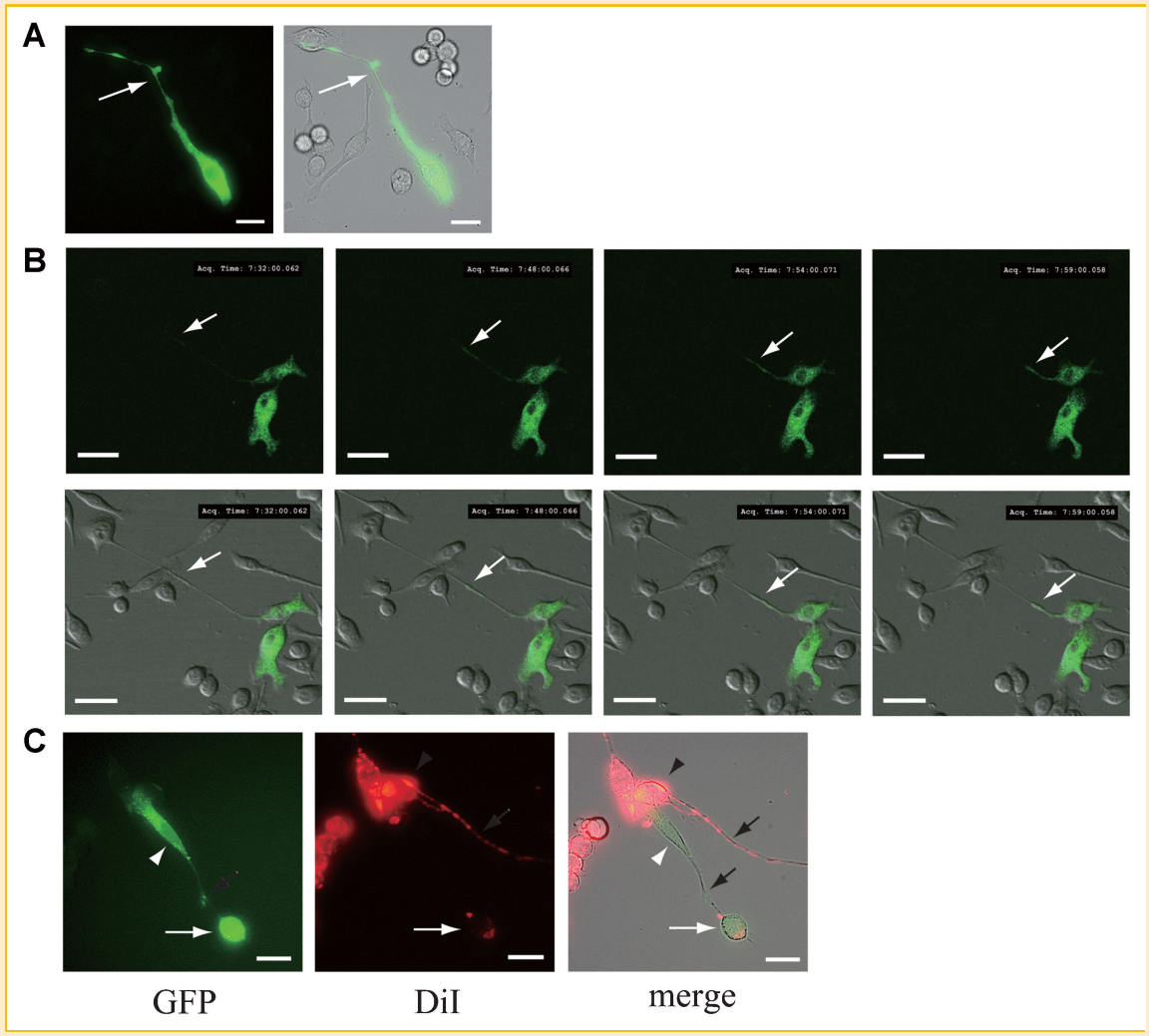

Fig. 6. Transportation of DC-STAMP proteins through TNTs formed among osteoclast precursors. A: Observation of DC-STAMP-GFP expressing cells. RAW-D cells were transfected with DC-STAMP-GFP expression vector. Right panel (phase contrast merged with GFP observation) is the same view of left panel. Arrows: TNT, Bars: 20 μm. B: Time-lapse analysis of DC-STAMP-GFP transportation through TNTs in RAW-D cells stimulated to form osteoclasts. After 48 h of RANKL stimulation, time-lapse analysis (every 1 min) was performed. Upper panels: GFP observation. Lower panels: Phase contrast views merged with GFP observation of the same area as the upper panels. Arrows: Migrating DC-STAMP-GFP fusion protein through TNT. Bars: 20 μm. C: A possible migration of DC-STAMP-GFP protein into osteoclast precursors labeled only with DiI in osteoclastogenesis. RAW-D cells expressing DC-STAMP-GFP and those labeled with DiI were mixed and cultured for forming osteoclasts. Fluorescence microscopic observation was performed at 50 h of culture. Left panel: detection of GFP, Middle panel: detection of DiI, Right panel: merge of the left panel, and middle panel. Bars: 20 μm. White arrow heads: DC-STAMP-GFP expressing preosteoclast. Black arrow heads: DiI-labeled preosteoclast. Black arrows: TNT formed among preosteoclasts. White arrows: DiI-labeled cells having DC-STAMP protein.

In our experiments, only 33% inhibition was obtained by M-Sec knockdown experiments whereas 98% inhibition was observed by Latrunclin B, suggesting the possible presence of other regulatory molecules in the TNT-mediated formation of osteoclasts in addition to M-Sec. During osteoclastogenesis, the maximum appearance of TNTs were just prior to the multinucleation in RAW-D culture system and that in bone marrow macrophages (mBMM) were more earlier than the multinucleation step, it could also be possible that osteoclast precursors utilize TNTs for communicating each other to accomplish efficient interaction in advance to the following specific fusion among osteoclast precursors.

Our current study shows the first evidence strongly suggesting an involvement of TNTs in osteoclastogenesis. Regulation of cell–cell communication would be one effective approach to regulate osteoclastogenesis.

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