Tropomyosin 4 Regulates Osteoclast Function through Actin Adhesion Structures

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Abstract

Osteoclasts, large, multinucleated cells which resorb bone, function to maintain bone degradation vital for maintenance and repair of bone tissue. Osteoclasts function by forming unique actin structures for migration, podosomes, and bone resorption, actin rings. The actin cytoskeleton also transports V-ATPases to the plasma membrane. Thus regulation of actin is central to the understanding osteoclast function. Tropomyosins are proteins that bind to actin and regulate other proteins association with actin. The purpose of this study is to investigate the role of tropomyosins, specifically tropomyosin 4, in osteoclasts.

Our research initially demonstrated the presence of eight tropomyosins in osteoclasts. Confocal imaging demonstrated that the eight tropomyosins were distributed in distinct locations. Of these eight tropomyosins, tropomyosin 4 colocalized with the interior faces of both actin rings and podosomes. To investigate the role of tropomyosin 4 expression was suppressed by RNA interference. The suppression led to reductions in actin ring thickness, bone resorption, and motility. Suppression also led to improper V-ATPase trafficking which was not due to a decrease in expression or complex formation. In addition, we produced stable cell lines that overexpressed tropomyosin 4. Overexpression resulted in abnormal podosomes that were thicker than normal and unusually distributed along with reduced cell motility. Further, actin ring formation was disrupted and bone resorption was abolished. Together the studies suggest that

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tropomyosin 4 regulates the adhesion structures of osteoclasts by stabilizing the actin in podosomes and actin rings and thus affecting osteoclast migration and resorption.

**Introduction**

Osteoclasts, bone degrading cells, and osteoblasts, bone forming cells, exist in functional equilibrium within the bone environment. If the balance of cellular activity is upset, too much bone formation or too little bone resorption occurs, resulting in osteoporosis or osteopetrosis. Though excessive bone resorption can lead to diseases such as osteoporosis, osteoclastic bone resorption is vital for bone growth, tooth eruption, fracture healing, and maintenance of blood calcium levels. Osteoclasts are large multinucleated cells formed by fusion of monocyte/macrophage precursors. This differentiation process occurs through the stimulation of precursors with macrophage colony stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B ligand (RANKL) (in reviews Teitelbaum 2007; Teitelbaum 2000).

Following a cyclical pattern of activity—migration, attachment, polarization, bone degradation, then migration—osteoclasts generate resorption pits in the bone matrix (Satel et al. 2004). To accomplish these processes, osteoclasts form specialized adhesion structures termed podosomes and actin rings. Podosomes are unique attachment structures formed by a core of actin (~1μm) that extends perpendicular to the substrate from the bottom of the cells and is surrounded by a variety of regulatory proteins (in reviews Jurdic et al. 2006; Linder and Kopp 2005; Linder and Aepfelbacher 2003). Podosomes are normally expressed only in cells of monocytic lineage such as macrophages and osteoclasts. These complexes bear some similarity to focal adhesions, but differ by possessing a half-life of 2-12 minutes rather than the focal adhesion half-life of 30 minutes; further, the actin core of podosomes is not present in focal adhesions. Podosome patterning follows specific steps in osteoclasts: first clustered podosomes appear within clouds of F-actin, followed by expansion of podosomes to form small rings, and finally formation of podosome belts at the periphery of the cell (Jurdic et al. 2006; Destaing et al. 2003). The
second actin structure used by osteoclasts for adhesion is called the actin ring, a large band of actin filaments that is formed only when osteoclasts are plated on bone substrate (Satel et al. 2004). Like the actin core of podosomes, the ring is composed of short microfilaments oriented perpendicular to the substrate. The actin ring allows the cell to form a tight attachment to the bone matrix to create a sealed extracellular microenvironment with an acidic pH and a high concentration of bone-degrading enzymes. The actin ring also allows the cell to polarize and form the ruffled border, a specialized cell membrane that contains V-ATPases, proton transporters that are responsible for creating the acidic pH needed for bone degradation (Teitelbaum 2007). Mice deficient in actin ring formation exhibit osteoporosis, the accumulation of too much bone, such as Src kinase knockout mice and V-ATPase a3 subunit knockout mice (Li et al. 1999; Lowe et al. 1993). In human, infantile osteopetrosis, which is fatal if not treated, is caused by a mutation in the a3 V-ATPase subunit causing the osteoclasts to not form actin rings (Komak et al. 2000). Additionally, the actin network within osteoclasts is responsible for the movement of the V-ATPases to the ruffled border plasma membrane (Lee et al. 1999). Thus, understanding the regulation of actin within osteoclasts is vital to our knowledge of how these cells function within the bone environment.

Tropomyosins are a class of proteins that stabilize microfilaments and regulate their activity by recruiting or inhibiting other regulatory proteins from associating with actin. Though they contain “myosin” in their name, they are not functionally similar to myosins, but rather function to regulate where particular myosin isoforms or other regulatory proteins can bind (Bryce et al. 2003; Ishikawa et al. 1989). Tropomyosins function by forming $\alpha$-helical dimers that bind to the grooves of F-actin. Over forty isoforms of four tropomyosin genes exist in mammals due to alternative splicing and promoters (Araya et al. 2002). Muscle cells tend to express only one or two different tropomyosin isoforms, while non-muscle cells tend to express six to eight. Tropomyosins typically consist of nine exons with alternative exons most often for exons one, two, six, and nine leading to differences mostly at the C- or N-termini of the proteins (Araya et al. 2002; Perry 2001). The differences between tropomyosin isoforms allow for
alternate tropomyosins to target different pools of actin within one cell to mediate distinct functions (Bryce et al. 2003).

The multiple roles of actin within osteoclasts have led us to study tropomyosins in osteoclasts. Our research demonstrated the presence of eight tropomyosins in osteoclasts in distinct expression patterns (McMichael et al. 2006). The two higher abundance tropomyosins, Tm5ab and Tm4, are localized to both podosomes and the actin ring of osteoclasts, while the lower abundance tropomyosins, Tm2/3, Tm5NM1, TmNM7, and TmNM11, are dispersed throughout the cell with no colocalization with the podosomes and only a slight colocalization with the actin ring. The various tropomyosins showed very little overlap between isoforms. This study has focused on using gain-of-function and loss-of-function methodologies coupled with functional assays to better understand the roles of tropomyosin 4 in osteoclasts.

**Results**

*Tropomyosin 4 localizes to the actin structures in osteoclasts*

Immunolocalization combined with confocal imaging showed that Tm4 localized to the podosome actin core (Figure 1A) and further analysis showed the localization to be on the interior tip of the podosome (data not showed). Along with the direct overlap of the actin podosome core, Tm4 localized to the interior face of actin ring in osteoclasts (Figure 1B). The localization of tropomyosin 4 suggested that it was involved in regulating these actin structures in osteoclasts.

*Suppression of tropomyosin 4 results in actin ring thinning and mislocalization of the V-ATPases*

To investigate the role of this protein further, Tm4 expression was knocked down via RNA interference in both the RAW 264.7 cell line (a macrophage cell line that can be differentiated in culture to form osteoclasts) and primary bone marrow derived osteoclasts. Small interfering RNA’s (siRNA) that specifically targeted Tm4 or a non-targeting control were transfected into osteoclasts and the expression levels (both RNA and protein) were analyzed over the following three days. Using an internal standard
with competitive RT-PCR we found that Tm4 RNA was suppressed one day following the transfection and remained suppressed through the three days analyzed (Figure 2). Protein levels were also assessed and found to decrease by about 50% one day after transfection and remained decreased over the same time period while the levels of GAPDH or beta actin did not change (Figure 2). Though only a 50% decrease in expression occurred, the transfection efficiency was over 95% and immunocytochemical analysis indicated that it was a uniform decrease in Tm4 in all the cells in the culture.
To assess the F-actin ring structures of osteoclasts with suppressed Tm4 levels, we collected confocal Z-stack images of cells plated on ivory labeled with fluorescent phalloidin. The actin ring thickness of untransfected, control siRNA transfected, and Tm4 siRNA transfected cells was measured at its thickest point. Figure 3A demonstrates the decrease in actin ring thickness in Tm4 suppressed cells from 9.3 um to 4.9 um in RAW osteoclasts and 9.5 um to 5.4 um in marrow osteoclasts (quantification Figure 3B). These data demonstrated that the 50% decrease in Tm4 protein led to a 50% decrease in the thickness of the actin rings, indicating that Tm4 plays a role in stabilizing actin rings.
In order for osteoclasts to resorb bone, they must create a tight adhesion via the actin ring and then localize enzymes such as V-ATPases within the actin ring- within the ruffled border. Since there was a difference in the ring thickness, we chose to assess whether the V-ATPases were correctly localized to the ruffled border. Immunolocalization was performed using antibodies that recognize subunits from each domain (V0 and V1 domains) of the V-ATPases in cells plated on ivory. In typical cells and the control cells, the V-ATPases were enriched within the actin ring (Figure 4 A/B upper panels). In about 20% of the Tm4 siRNA-treated cells, the V-ATPases failed to be enriched within the actin ring (Figure 4 A/B lower panels). Protein levels were measured by Western blot and showed that there were not significant differences in the protein levels of the V-ATPases compared to the control (data not shown). In addition, V-ATPases from metabolically S35 labeled cells were immunoprecipitated to show that the complex still properly formed in the siRNA-treated cells (data not shown). Therefore, the difference in V-ATPase localization between control and Tm4 siRNA-treated cells seems to be from improper V-ATPase trafficking and not expression or complex formation.
Fig. 4. Mislocalization of V-ATPases. Confocal images of cells on ivory were compared to assess the localization of V-ATPases. Antibodies were used to the E1 subunit (A) and a3 subunit (B) of V-ATPases. In about 20% of siRNA Tm4 cells, the V-ATPases were not concentrated within the actin ring (lower pictures).

Suppression of tropomyosin 4 leads to a decrease in osteoclastic bone resorption and motility

The Tm4 siRNA-treated cells were functionally evaluated through bone resorption and motility assays. The decrease in the actin ring thickness and the mislocalization of V-ATPases suggests that suppression of Tm4 will cause disruption of bone resorption activity. To measure resorption, control and Tm4 siRNA-treated cells were plated on synthetic bone substrate for three days and the area resorbed was measured. Figure 5A illustrates that Tm4 siRNA-treated cells produced both decreased numbers of clearings as well as lower area per clearing, leading to a decrease in the total area resorbed. Additionally, Tm4 siRNA-treated cells generated clearings with an altered shape. When a normal osteoclast resorbs bone, the area beneath the ring is resorbed and typically tends to be a rounded clearing. However, the Tm4 siRNA-treated cells generated incompletely resorbed clearings (Figure 5B). Because altered cell motility may play a role in this process, transwell migration assays were performed to evaluate the motility of Tm4 siRNA-treated cells. The number of cells that migrated from the top to the bottom of the chamber was decreased by about 50% relative to control cells (Figure 5C). Although Tm4 siRNA-treated cells still displayed podosomes, we also found unusual long actin fibrils only where Tm4 was absent, which could account for the decrease in motility.
Overexpression of tropomyosin 4 results in abnormal actin structures

Because tropomyosin 4 suppression led to a decrease in the actin ring thickness and altered actin structures, we overexpressed Tm4 to see if it would also alter the actin structures in osteoclasts. Stable cells lines were created with the cDNA for Tm4 or an empty vector as a control and individual clones were picked that overexpressed Tm4 to varying degrees (Figure 6A). By Western analysis the best clones expressed only a two-fold increase of Tm4, yet all the clones tested generated altered actin structures displayed in both figures 6 and 7. The promoter for the vector used was human elongation factor 1 alpha. When Tm4 was overexpressed with a stronger CMV promoter, the cells died- indicating that the cells could not tolerate a high overexpression of Tm4. Initially the overexpressing cells were assessed on glass in their migrating phenotype. Overexpressing cells had brighter F-actin labeled podosomes that were unusually distributed throughout the cell rather than near the cell edge as shown in the control (Figure 6B). Because the overexpressing cells had such bright podosomes, we used Z-stack imaging to measure the height of the podosomes. Compared to the control, the height of the podosomes increased from 2 um to 3.5 um (Figure 6C). Along with the phenotypic analysis, we used transwell migration assays to
determine if the abnormal podosomes were affecting migration. Migration decreased 65% in overexpressing cells (Figure 6D).

Fig. 6. Overexpression migration phenotype. A. Western blots of four clones that overexpress Tm 4 with no change in GAPDH or beta actin. B. Mislocalized podosomes seen by phalloidin stained cells plated on glass. C. Podosome height was measured via Z-stack images and demonstrated an increase in the height of podomes in overexpressing cells. D. Motility was measured with transwell migration assays with the average +/- s.d. of 3 experiments.

To assess the bone phenotype of the overexpressing cells, they were plated on ivory. Control cells were able to polarize and form proper actin rings, while overexpressing clones were unable to form actin rings. Overexpressing cells formed patches of actin where the actin ring should have been at the base of the cell (Figure 7). Together with the abnormal actin at the base of the cell, the patches of actin were formed around the cell membrane of the cells. As expected, under normal conditions where the cells were plated on the synthetic bone substrate for three days, the overexpressing cells were unable to resorb bone. For bone resorption data, cells were plated on the synthetic bone substrate for three days. Since these cells did not resorb any of the substrate, while the control resorbed normal amounts, the experiment was repeated and the cells were left on the synthetic bone substrate for eight days. The overexpressing clones resorbed approximately 9% of control levels after this longer time period.
Fig. 7. Abnormal actin structure of overexpressing Tm4 cells on bone. Osteoclasts that overexpressed the control plasmid or Tm4 were plated on ivory. The control cells form normal actin ring structures at the base of the cell (left) while the Tm4 cells formed abnormal actin patches at the base of their cells (right).

Discussion

These studies strongly suggest that Tm4 is playing a role in stabilizing osteoclast adhesion structures and is thus affecting resorption and motility in osteoclasts. Our initial studies found that eight tropomyosins are expressed in osteoclasts. Tropomyosin 4 was the first tropomyosin we picked to study since it is easier than the other isoforms to manipulate genetically being the only product of the delta gene. Tm4 is also unique from the other osteoclast tropomyosins since it is the only tropomyosin that is localized to the actin core of the podosome- other tropomyosins surround the core or are near the core, but none of the other tropomyosins in osteoclasts overlap the core. The localization further indicated to use that Tm4 may be playing a stabilizing role for the actin- which was supported by these studies.

Our preliminary studies indicate that Tm4 only binds one protein directly (data not shown), but it may be preventing many additional actin regulatory proteins from binding the actin within the actin ring and podosomes. When there is less Tm4, other proteins such as gelsolin or cofilin can bind and change the actin structures in osteoclasts. Tropomyosins have been shown to regulate the access of gelsolin and cofilin to actin (Ishikawa 1989; Bernstein and Bamberg 1982). Additionally, since endogenous Tm4
forms a cap on the actin ring, it is possible that Tm4 may be holding specific actin ring regulatory proteins in the correct conformation needed for proper ring formation. Studies have found that the Arp2/3 complex localizes to the bottom half of the actin ring while we found Tm4 localizes to the top half of the actin ring (Hurst et al. 2004). Further studies are being conducted to look at the change in the distribution of various actin regulatory proteins in response to either under- or overexpression of tropomyosin 4 to help us better understand how tropomyosin is functioning in osteoclasts.

Methods and Materials

Osteoclast culture—Murine osteoclasts were generated either from the macrophage cell line RAW264.7 or from mouse bone marrow. To generate RAW264.7-derived osteoclasts, macrophage precursors were cultured for 5-7 days in DMEM containing 100 ng/ml of a GST-RANKL fusion protein. For the primary cell preparation, marrow cells from male Swiss-Webster mice were incubated overnight in [alpha] MEM containing 20 ng/ml M-CSF. The next day and additional 5-6 days, the cell were incubated in [alpha] MEM containing 20 ng/ml M-CSF, and 100 ng/ml GST-RANKL.

Antibodies and Western analysis—For detection of Tm-4, a rabbit polyclonal was purchased. Loading control antibodies to GAPDH and beta actin, both mouse monoclonal antibodies, were used. Antibodies against V-ATPase subunits E1 and a3 were generated in this laboratory. For Western analysis, osteoclast lysates used standard methodology, and were detected with SuperSignal West Pico Chemiluminescent reagents.

Competitive RT-PCR of Tm-4 mRNA—To determine Tm-4 mRNA expression levels by RT-PCR, primers were created that corresponded to sequences within the murine cDNA. For an internal standard, a cDNA was created that corresponded to an expected PCR, but contained an internal deletion of 17%. This product was transcribed in vitro using the MAXIscript system, and 10 pg of the resulting RNA was added to 1 ug of osteoclast total cellular RNA prior to reverse transcription and PCR. The resulting RT-PCR products were run in a 2% agarose gel to visualize the relative intensities of the bands. Quantification of bands was performed with a Chemi-doc supplied with Quantity One software.

Immunocytochemistry and Microscopy—Osteoclasts were cultured either on glass coverslips or thinly-cut ivory slices. Cells were fixed and permeabilized and primary antibodies were detected using Alexa-labeled secondary
antibodies. F-actin was visualized using Alexa-labeled phalloidin. Cells were visualized using a confocal
microscope (Campus Microscopy and Imaging Facility, The Ohio State University). Actin ring and podosome
thickness was determined by generating multiple Z-stack images and measuring these rings at their thickest points.

Knockdown and overexpression of Tm-4—To knockdown Tm-4, siRNA’s were designed and synthesized by
Ambion. On day 5 of RAW264.7 differentiation, a 75 nM solution of siRNA’s or an equal volume of a control
siRNA was added to Lipofectamine 2000. The medium was replaced after five hours. For immunocytochemical
analysis, the cells were scraped and replated on ivory slices or glass coverslips immediately following the
transfection. For RNA analysis, total cellular RNA was harvested one to three days after the transfection with RNA-
Bee. For protein analysis, whole cell lysates were harvested one to three days post transfection with M-PER.

Bone marrow-derived osteoclasts were transfected via electroporation. On day five of differentiation in culture,
cells were scraped, pelleted, and resuspended in siPORT buffer. The cells were electroporated at 250V/ 50uF with
the siRNA solution or control siRNA, then plated in standard differentiation medium on glass or ivory for
immunocytochemistry, or plastic for RNA and protein analysis. Both transfection rates were over 95%.

For generation of a Tm-4 expression plasmid, an I.M.A.G.E. clone corresponding to the murine form was used.
A fragment containing the entire coding region was subcloned into the eukaryotic expression vector pEF6/V5-His.
This construct was stably transfected into RAW264.7 macrophages; the empty pEF6/V5-His vector was stably
transfected into cells as a control. Cells were maintained in 3 ug/ml blasticidin for selection.

Osteoclast resorption and motility assays—Five days after initial RANKL stimulation, osteoclasts were
transfected and immediately plated on BD BioCoat Osteologic Discs. Control and siRNA-treated cells were kept on
the discs for three days. The cells were removed and resorption pits were assayed under low magnification, and
quantifying resorbed areas with SigmaScan Pro 5.0 software. Equal numbers of images were compared among test
groups.

Motility was measured by the use of 8.0 um pore Transwell migration chambers. Five days after RANKL
stimulation or immediately following transfection of RAW or marrow cells, the cells were scraped and replated on
the upper side of the membrane. After 48 hours, the cells were stimulated overnight to migrate by the addition of 40
ug/ml osteopontin peptide to the bottom of the well. Cells were fixed and stained for tartrate resistant acid
phosphatase.
References


