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Expression and Function of Transmembrane-4 Superfamily (Tetraspanin) Proteins in Osteoclasts: Reciprocal Roles of Tspan-5 and NET-6 during Osteoclastogenesis

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ABSTRACT

Background: Osteoclasts are bone-resorbing multinuclear polykaryons essential for bone remodeling, formed through cell fusion of mononuclear macrophage/monocyte lineage precursor cells upon stimulation by the RANK/RANKL system. Recent studies have revealed that a family of tetraspanin proteins, such as CD9, is critically involved in the cell fusion/polykaryon formation of these cell types. Until now, however, there is limited knowledge about the types of tetraspanins expressed in osteoclasts and their precursors.

Methods: The expression of different tetraspanin proteins in a monocyte/macrophage-lineage osteoclast precursor cell line, RAW264.7, was cyclopedically investigated using RT-PCR with specific primers and quantitative real-time RT-PCR. The function of two kinds of tetraspanins, Tspan-5 and NET-6, whose expression pattern was altered by RANKL stimulation, was examined by transfecting gene-specific short-interfering RNAs into these cell types.

Results: Of the 17 tetraspanins in mammalian hematopoietic cells, RAW264.7 cells express mRNA for 12 different kinds of tetraspanins, namely, CD9, CD37, CD53, CD63, CD81, CD82, CD151, NAG-2, NET-6, SAS, Tspan-3, and Tspan-5. Interestingly, during their maturation into osteoclasts upon RANKL stimulation, the transcript for Tspan-5 is up-regulated, whereas that for NET-6 is down-regulated. Targeted inhibition of Tspan-5 by using gene-specific RNA interference suppressed RANKL-induced cell fusion during osteoclastogenesis, whereas inhibition of NET-6 augmented the osteoclastogenesis itself. These results suggest that Tspan-5 and NET-6 have a reciprocal function during osteoclastogenesis, *i.e.*, positive and negative regulation by Tspan-5 and NET-6, respectively. RANKL regulates osteoclastogenesis by altering the balances of these tetraspanin proteins.

Conclusions: These data indicate that a diversity of tetraspanins is expressed in osteoclast precursors, and that cell fusion during osteoclastogenesis is regulated by cooperation of distinct tetraspanin family proteins such as Tspan-5 and NET-6. This study indicates that functional alterations of tetraspanin family proteins may have therapeutic potential in diseases where osteoclasts play a major role, such as rheumatoid arthritis and osteoporosis.

KEY WORDS

cell fusion, NET-6, osteoclast, tetraspanin, Tspan-5

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INTRODUCTION

Osteoclasts are bone-resorbing multinuclear giant cells which are differentiated from macrophage/monocyte lineage precursors.¹⁻³ In patients with bone-resorbing disorders, such as rheumatoid arthritis and osteoporosis, increased numbers of activated osteoclasts cause the bone destruction seen in such diseases.^{4,5} It has been established that osteoclastogenesis is mainly regulated by the RANK (the receptor activator of nuclear factor κ B)/RANKL (RANK ligand) system. However, the mechanism whereby osteoclast precursors fuse with each other to form a polykaryon was until recently a mystery.^{6,7}

The tetraspanins, also referred to as the transmembrane-4 superfamily (TM4SF), are characterized by their four transmembrane domains and comprise at least 20 distinct members in mammalian hematopoietic cells, including CD9, CD37, CD53, CD63, CD81 and CD151.⁸⁻¹¹ One of the tetraspanins, CD9, is a membrane glycoprotein (about 25 kDa), which is expressed in a variety of cell types, and has been implicated in the regulation of cell fusion during osteoclastogenesis.^{12,13} However, virtually nothing is known about the expression of different types of tetraspanin proteins in osteoclasts, except for CD9. Because multinucleation (*i.e.*, polykaryon formation) is a characteristic of osteoclasts that distinguishes them from all other somatic cells, the identification of the molecules responsible for this event is expected to lead to the development of novel therapeutic agents for controlling bone-resorbing diseases. To see the whole views of regulation of osteoclast formation with tetraspanin family proteins, it is necessary to encompass the cyclopedic lists of tetraspanin expressed in osteoclasts.

In this study, using RT-PCR methods with transcript-specific primers, we investigated the mRNA expression of the tetraspanin family proteins expressed in an osteoclast precursor cell line, RAW 264.7. Furthermore, in order to identify the functions of two of the tetraspanin expressed proteins, we examined the effect of targeted inhibitions by using short interfering RNAs.

METHODS

CELL CULTURE

RAW264.7, a murine macrophage/monocyte lineage cell line, was obtained from ATCC (ATCC#: TIB-71), and cultured with α -MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, UT, USA) and penicillin/streptomycin (Invitrogen).¹³ RAW264.7 can fully differentiate into mature osteoclast-like cells by stimulation with RANKL, and is therefore commonly used for a model system of *in vitro* osteoclastogenesis.¹⁴ In order to differentiate the cells into multinuclear osteoclasts, 50 ng/ml RANKL (PeproTech,

Rocky Hill, NJ, USA) was added to the medium and incubated for 3–5 days.

RT-PCR AMPLIFICATION

Total RNAs of RAW264.7 cells were extracted using TRIzol™ reagent (Invitrogen) and cDNAs were synthesized with SuperScript III reverse transcriptase (Invitrogen), according to the manufacturers' protocol. To eliminate genomic DNA contamination, we treated the RNA samples with DNaseI (Takara, Kyoto, Japan) before cDNA synthesis. Specific primer pairs (listed in Table 1) were used to amplify cDNA. PCR amplification was performed for 25 cycles at 95°C for 45 seconds, at 55°C for 30 seconds, and at 72°C for 1 minute. The products were electrophoresed on 2% agarose gel. The amplified PCR products were confirmed by their nucleotide sequencing using ABI Dye terminator cycle sequencing with the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

QUANTITATIVE REAL-TIME PCR

Relative quantification with real-time RT-PCR was performed using an ABI PRISM 7900 (Applied Biosystems) with an Assay-on-Demand TaqMan probe and relevant primers (Tspan-5, Assay ID #Mm00497960_m1; NET-6 (Tspan-12), Assay ID #Mm00481226_m1; GAPDH, Assay ID #Mm99999915_g1), according to the manufacturer's instructions. Isolation of total RNA from RAW264.7 cells and cDNA synthesis were performed as described above. Each sample was assayed in triplicate and the median threshold cycle (Ct) value was used to calculate the relative concentration of transcripts. Values were normalized against the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

RNA INTERFERENCE

A small interfering RNA duplex (siRNA) targeting Tspan-5 and NET-6, and the control RNA duplex were synthesized by, and purchased from, B-Bridge International Inc. (Sunnyvale, CA, USA): Tspan-5 siRNA, 5'-GGAUCAAGACCAGCUGUATT-3', and NET-6 siRNA, 5'-GCUAAUUGGGGAGCUGUAAAATT-3'. The synthesized 21-mer sense and antisense RNA strands were hybridized. The transfection of hybridized siRNAs into RAW264.7 cells was performed using LipofectAmine2000 (Invitrogen) according to the manufacturer's instructions. After transfection, the cells were incubated for 3 days in α -MEM supplemented with 10% FCS and antibiotics. Then, the cells were fixed in citrate acetone fixative, stained for tartrate-resistant acid phosphatase (TRAP) with a staining kit (Sigma-Aldrich, St. Louis, MO, USA), and observed by an inverted differential interference microscope (IX-71, Olympus, Tokyo, Japan), equipped with a 20X objective (UPLSAPO, Olympus; N.A. ~0.75). The picture image was acquired on-line through a

Table 1 Primer pairs used for RT-PCR to detect mRNA for different types of tetraspanin superfamily proteins. The expected molecular weight in base pairs (b.p.) is indicated. As a control the presence of mRNA for GAPDH was detected.

	Forward (5'-3')	Reverse (5'-3')	b.p.
CD9	CTTGCTATTGGACTATGGCT	GTCCGAGATAAACTGCTCCA	405
CD37	TTTGTGGGTTTGTCTTCGT	GGGAGAAAAAGAGCTTATCA	463
CD53	TATGGAGTACTCTTCCGTAA	AGCACCTGTATCACACATAC	476
CD63	TTCAGGTTGTCTTGAAGCAG	ATGTTGATGCAGCAAGAATC	415
CD81	TACCTGGAACCTGGGAAACAA	GCTACCACAATGGCTGCAAT	503
CD82	TACAAACCTCATCCAGCTCG	TCTTCACAATGAGCTGGTTG	436
CD151	AGTGACTACATTAGTCTGCT	TAGTCTTACAGCAGCTGTCA	463
CD231	TGTTCTGGCTGAGCTTGTT	ACTGATTGGCAGTGATGAAC	449
NAG-2	ATCATCCTCATTTCATCCT	TTTACCACCTGGCAGTACAT	564
NET-2	ATCAGTGTCTTGGCAGTTTC	AGTAATGGTGAGAATCATGG	654
NET-5	TTGATCATCCTGCTGGCAGA	GCACGTGCTTGTGTCA	344
NET-6	TTTGGGCTGATCTCCAGTCT	CAAACCTCAAACCTCTCCA	397
SAS	GTGGTGTCTAGCATTACAT	GTCTGAATGTTTAAGGAACC	393
Tspan-2	GGAGGTACCATGAAGGATT	GGTTTCAATTTGTGCGATGC	427
Tspan-3	CTATGTCTTCATCACCTATG	TTCACAACAAGAGCCTCACA	523
Tspan-5	AAGGGTCCTGAAGTCAGTTG	TGTCCTGTAGCCACTTCTCA	676
Tssc6	TACTGATCACCACCTTCTA	GAAGCAACTGTAGTGTGT	556

cooled CCD (ORCA 3CCD; Hamamatsu Photonics, Hamamatsu, Japan) and processed with data acquisition software (Aquacosmos; Hamamatsu Photonics). For the evaluation of osteoclastogenesis, nuclei of multinucleated (more than four nuclei) TRAP-positive cells were counted using light microscopy. More than 1000 nuclei in ten visual fields from three independent experiments were counted. The reduction of the amount of mRNAs in the targeted cells by these siRNAs was assessed by conventional RT-PCR using their specific primers, as described above. The expression of NFATc1 was also assessed by conventional RT-PCR as previously described.¹⁵

STATISTICS

All results are expressed as the mean \pm standard error (s.e.m.) obtained from *n* experiments. Statistical differences were evaluated by unpaired Student's *t*-tests. Statistical probability values of *p* < 0.05 were considered to indicate significant differences.

RESULTS

EXPRESSION PROFILE OF TETRASPANIN SUPERFAMILY PROTEINS IN AN OSTEOCLAST PRECURSOR CELL LINE

In the RT-PCR analysis of total RNA extracted from RAW264.7 cell lines cultured in the absence or presence of RANKL-stimulation, oligonucleotide primers for 12 kinds of tetraspanin superfamily proteins showed an amplification of products of expected sizes (Fig. 1A): CD9, CD37, CD53, CD63, CD81, CD82, CD151, NAG-2, NET-6, SAS, Tspan-3, and Tspan-5. These amplified products were confirmed by their nucleotide sequencing. As a control, the presence of

mRNA for GAPDH was examined, and it was found to be present at the expected molecular sizes (Fig. 1A).

Upon RANKL stimulation, mRNA for Tspan-5 was shown to be up-regulated, whereas that for NET-6, on the other hand, was down-regulated (Fig. 1A). On the other hand, the expression of other tetraspanins, *i.e.*, CD9, CD37, CD53, CD63, CD81, CD82, CD151, NAG-2, SAS, and Tspan-3 were not significantly altered at least in mRNA levels.

Expression of Tspan-5 and NET-6 was also examined quantitatively by real-time RT-PCR using a TaqMan probe and relevant primers (Fig. 1B). Upon stimulation of osteoclastogenic RANKL signaling, Tspan-5 mRNA was promptly increased to 2.5-folds in 1 day after, and the up-regulation was sustained for up to 4 days (Fig. 1B, *upper panel*). On the other hand, the transcription of NET-6 was down-regulated to about 1/5 one day after RANKL stimulation but its level was gradually recovered (Fig. 1B, *lower panel*). This result may suggest that the suppression of NET-6 expression is important in the earlier phase of osteoclastogenic cell fusion.

TARGETED INHIBITION OF TWO TETRASPANIN PROTEINS, TSPAN-5 AND NET-6

Of the 12 tetraspanin proteins expressed in RAW 264.7 cells, we took special notice of Tspan-5 and NET-6, because their expressions are changed in response to RANKL stimulation, suggesting their significant roles in osteoclastogenesis. To investigate the role of these tetraspanin proteins on osteoclastogenesis, we examined the effect of inhibiting their functions with short interfering RNAs (siRNAs) targeting mouse Tspan-5 and NET-6 (Fig. 2). The siR-

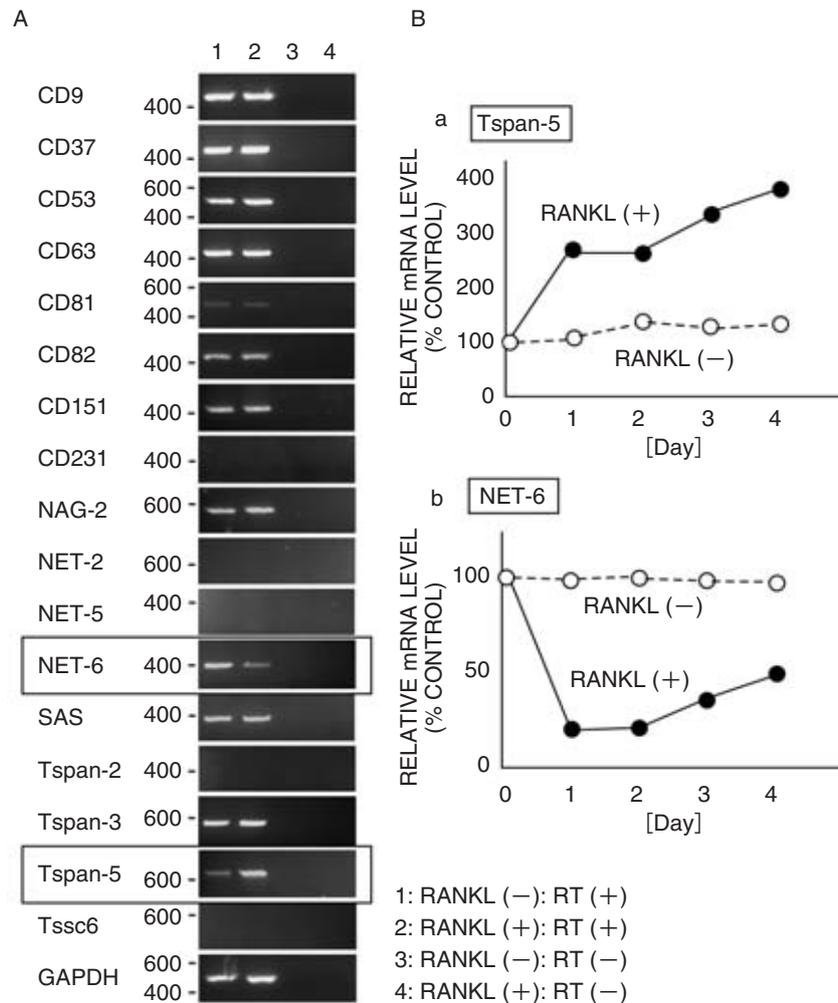


Fig. 1 (A) RT-PCR analysis of mRNA encoding 17 different kinds of mammalian tetraspanin superfamily proteins expressed in an osteoclast precursor cell line, RAW264.7, cultured in the absence (RANKL (-)) or presence (RANKL (+)) of RANKL. As a control, mRNA for GAPDH was detected. RT (+), PCR with synthesized cDNA; RT (-), negative controls without reverse transcriptase. The numbers on the left side indicate the positions of molecular weight markers in base pairs (b.p.) (B) Time-courses of expression of Tspan-5 (a) and NET-6 (b), examined using quantitative real-time RT-PCR analysis. RAW264.7 cells were cultured in the presence (filled circle) or absence (open circle) of RANKL for up to 4 days.

NAs and its control RNA duplex were generated and transfected into RAW264.7 cells. According to the basic experiments for expressing green fluorescent protein, the transfection efficiency in these cell types was estimated to be approximately 40–60% (data not shown).¹³ The reduction of the amount of Tspan-5 and NET-6 mRNAs in the targeted cells by these siRNAs was assessed by RT-PCR using gene specific primers (Fig. 2A). These siRNA potently inhibited the mRNA expression to about 50% in Tspan-5 (Fig. 2Aa) or to about 60% in NET-6 (Fig. 2Ab) at the transcript levels. On the other hand, the transcription of

NFATc1, a transcription factor crucial for osteoclastogenesis¹⁶ (Fig. 2A, lower panels), as well as those of other osteoclast markers, such as c-fos, RANK and α integrin (data not shown), were not significantly altered, suggesting that neither Tspan-5 nor NET-6 are involved in general osteoclastogenic signaling cascades.

Next, the transfection cells were cultured for 3 days in the presence of RANKL to induce differentiation. The results showed that the formation of osteoclast-like polykaryons was reduced by the inhibition of Tspan-5 expression (Fig. 2Bb). Interestingly,

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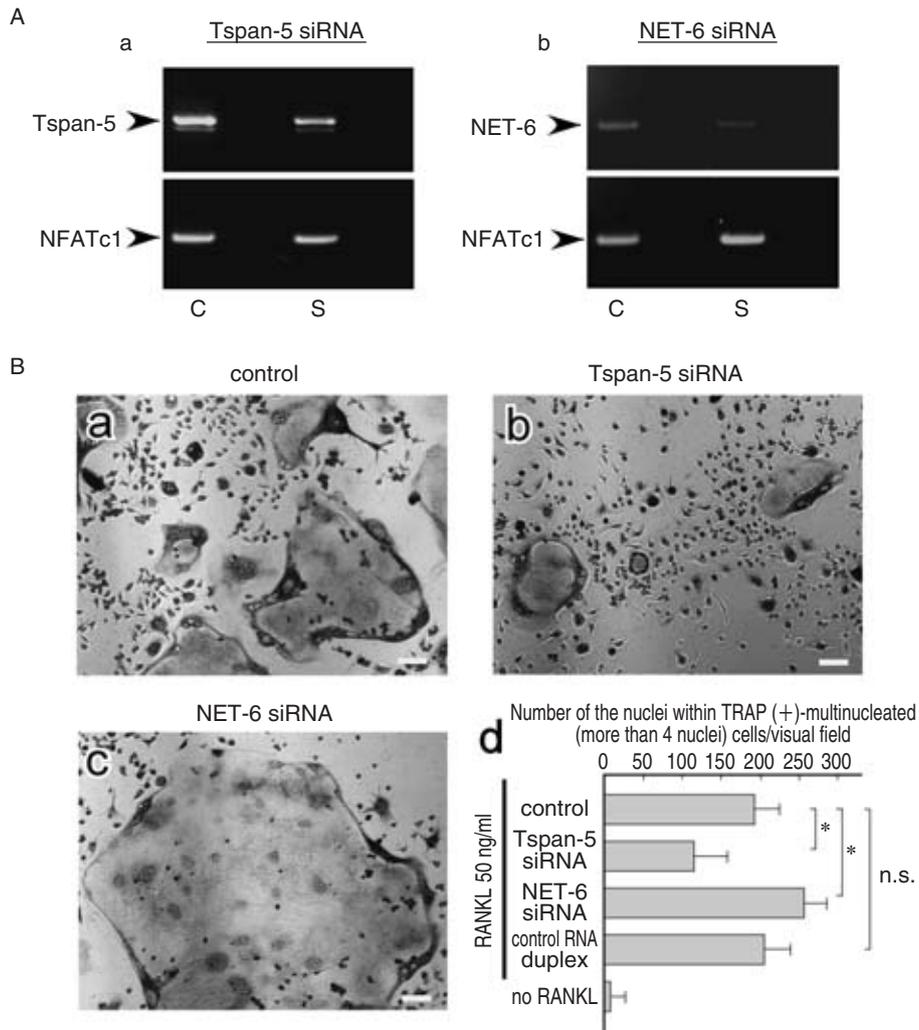


Fig. 2. Targeted inhibition of Tspan-5 and NET-6 using gene-specific short-interfering RNA (siRNA). **(A)** Reduction of mRNAs of Tspan-5 **(a)** and NET-6 **(b)** assessed by RT-PCR analysis. Expression of NFATc1, one of the transcriptional factors crucial for osteoclast maturation, was not significantly altered at the transcriptional level by the siRNA treatment. **(B)** Representative images of RANKL-treated RAW264.7 cells **(a)** in control conditions, and **(b)** transfected with Tspan-5 siRNA and **(c)** with NET-6 siRNA. Compared with the control conditions **(a)**, the decrease of Tspan-5 transcript by siRNA inhibits osteoclastogenesis **(b)**, whereas that of NET-6 prominently enhances osteoclastogenesis and the formation of giant polykaryons **(c)**. Scale bars represent 50 μ m. **(d)** Number of nuclei within TRAP (+)-multinucleated cells (cells with more than 4 nuclei) per visual field. Error bars represent \pm s. e. m. * represents $p < 0.05$. n.s., not significantly different. More than 1000 nuclei in ten visual fields were counted, and the results were essentially consistent in three independent experiments.

on the other hand, upon the decrease in NET-6 expression, the cells were found to differentiate into TRAP-positive osteoclasts to form giant polykaryons more efficiently than the cells in control conditions (Fig. 2Bc). These results clearly demonstrated the reciprocal function of the two tetraspanin proteins, Tspan-5 and NET-6, in osteoclastogenesis; *i.e.*, positive regulation by Tspan-5 and negative regulation by

NET-6. It is suggested that RANKL stimulation facilitates osteoclastogenesis by enhancing the expression of the positive regulator, Tspan-5, and by inhibiting expression of the negative regulator, NET-6.

DISCUSSION

One of the major characteristics of bone-resorptive osteoclasts is cell fusion and multinucleation during

their maturation into giant polykaryons. Tetraspanins are well known to be critically involved in the process of cell fusion, which includes osteoclastogenesis.^{12,13,16-20} Nevertheless, the tetraspanin superfamily consists of at least 20 subtypes, and it is still unsolved which subtypes are specifically responsible for osteoclastogenesis. In the current study, we revealed the redundant expression of mRNAs for 12 members among 17 mammalian hematopoietic tetraspanins, such as CD9, CD37, CD53, CD63, CD81, CD82, CD151, NAG-2, NET-6, SAS, Tspan-3, and Tspan-5. We found that two of the tetraspanin proteins, Tspan-5 and NET-6, are dynamically regulated by osteoclastogenic RANKL signaling at the transcriptional level. This case shows a marked contrast to that of CD9, whose membrane localization (especially to a specific membrane microdomain called "lipid raft") is regulated by RANKL stimulation.¹³

Regarding the role of tetraspanins in osteoclastogenesis, there is some controversy. Although most previous reports have indicated that tetraspanins are crucial for the formation of polykaryons including osteoclasts,^{13,17-21} one report has shown that tetraspanins such as CD9 and CD81 do not promote but rather inhibit multinucleated osteoclast formation, by using CD9- and CD81-global knockout mice.¹² Given the severe redundancy and crucial role of tetraspanins in osteoclasts, we can reasonably speculate that some other tetraspanins, *i.e.*, CD37, CD53, CD63, CD82, CD151, NAG-2, NET-6, SAS, Tspan-3, and Tspan-5, are up-regulated and possibly compensate the losses-of-function of CD9 and CD81.

Of the 12 expressed tetraspanins, two molecules, Tspan-5 and NET-6, are worthy of note, because their mRNA expressions are altered upon stimulation by RANK/RANKL signaling, and they have reciprocal roles in osteoclastogenesis. This case shows a marked contrast to that of CD9, whose membrane localization (especially to a specific membrane microdomain called "lipid raft") is regulated by RANKL stimulation.¹³ Tspan-5, whose expression in osteoclast precursors was up-regulated by RANKL and thus has a positive regulator of osteoclast maturation, has previously been shown to be abundantly expressed in the brain and to be involved in the development of the central nervous system.²²⁻²⁴ It has also been reported that Tspan-5 is expressed in unique natural killer (NK) cell subsets detected in the uterine decidua in early pregnancy.²⁵ NET-6, also referred as Tspan-13, a negative regulator of osteoclastogenesis, is known as a kind of aberrantly expressed cell marker in breast cancers, and is thought to play a role in mammary neoplasia.²⁶ Despite the studies revealing the expression profiles, the specific function of these tetraspanins, Tspan-5 and NET-6, remained unsolved.

It is an intriguing, but still unanswered, question how these different tetraspanin proteins exhibit con-

tradictory functions in cell fusion. In general, tetraspanin proteins are known to function as organizers of multimolecular membrane complexes ("tetraspanin web"), by composing a certain lipid microdomain, designated as a "tetraspanin-enriched microdomain (TEM)".^{10,11} Then it can be suggested that Tspan-5 and NET-6 form their webs cooperatively or respectively on the cell membrane and recruit unknown *cis*-interacting partners important for osteoclast cell fusion. Otherwise, they may behave competitively to unknown ligands for stimulating cell fusion. Further studies are needed to clarify the molecular functions of tetraspanin proteins in this scheme.

In conclusion, we revealed the redundant expression of the tetraspanin superfamily proteins in osteoclast precursor cell lines CD9, CD37, CD53, CD63, CD81, CD82, CD151, NAG-2, NET-6, SAS, Tspan-3, and Tspan-5, and specific reciprocal functions of Tspan-5 and NET-6. This finding may contribute to an overall understanding of tetraspanin-regulated cell fusion during osteoclastogenesis, which has recently emerged as a novel therapeutic target for bone-resorbing disorders.

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