Journal of Osteoporosis & Physical Activity

Kotake et al., J Osteopor Phys Act 2017, 5:1
DOI: 10.4172/2329-9509.1000194

Research Article Open Access

Bone Analysis of T-Cell Leukaemia Translocation-Associated Gene (TCTA) Transgenic Mice and Conditional Knockout Mice: Possibility that TCTA Protein Expressed on Osteoclasts Plays a Role as a Novel 'Coupling Factor' *In Vivo*

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Received date: March 16, 2017; **Accepted date:** March 23, 2017; **Published date:** March 29, 2017

Abstract

T-cell leukemia translocation-associated gene (TCTA) protein is expressed ubiquitously in normal human tissues. However, its function has not been clarified. In 2009, we demonstrated that TCTA protein play an important role in human osteoclastogenesis and pit formation of mature human osteoclasts inducing the fusion process of osteoclastogenesis *in vitro*. In the current study, to clarify the role of TCTA protein *in vivo*, we generated TCTA gene transgenic, both systemic and osteoclast-specific, mice and osteoclast-specific conditional knockout mice and then analyzed their bones. Surprisingly, in the conditional knockout mice, bone volume decreased despite inhibited osteoclastogenesis. According to these findings, we speculated that TCTA protein expressed on osteoclasts plays a role as a 'coupling factor' *in vivo*.

Keywords: Bone analysis; T-cell; Knockout mice; TCTA; Transgenic mice; Osteoclasts; Coupling factor

Introduction

In 2009, we demonstrated that a novel peptide, GQN, derived from the extracellular domain of T-cell leukemia translocation-associated gene (TCTA) protein inhibits both RANKL-induced human osteoclastogenesis and pit formation of mature human osteoclasts *in vitro* [1]. We also demonstrated that TCTA protein *per se* expressed on human monocytes and osteoclasts play an important role in the differentiation of osteoclasts inducing the fusion process of osteoclastogenesis [1].

The 29-mer peptide, GQNGSTPDGSTHFPSWEMAANEPLKTHRE from TCTA protein that we designated "peptide A" most potently inhibited human osteoclastogenesis. We finally hypothesized that the interaction of TCTA protein and its counterpart protein play an important role in human osteoclastogenesis and that the interaction is inhibited by peptide A [1].

We then planned to perform the experiment *in vivo* using mice. We constructed mouse "peptide A" and 2 other peptides including GQN from the mouse sequence of TCTA. In contrast to our expectation, however, these peptides did not inhibit mouse osteoclastogenesis from mouse bone marrow cells *in vitro* [1]. In contrast, human peptide A inhibited monkey osteoclastogenesis using peripheral monocytes from crab-eating monkeys [2].

TCTA protein is expressed ubiquitously in normal human tissues; however, its function has not been clarified. In 1995, Aplan et al. cloned a novel gene at the site of a t (1;3) (p34;p21) translocation breakpoint in T-cell acute lymphoblastic leukemia and they designated this gene as TCTA [3]. TCTA mRNA is expressed ubiquitously in normal tissues. A short open reading frame encodes a protein of 103 amino acid residues, Mr 11,300, without strong homology to any previously reported proteins [3]. Genomic Southern blots demonstrated a reduced TCTA signal in three of four small cell lung cancer cell lines, suggesting the loss of one of the two copies of the gene [3]. In 2005, Rual et al. reported that TCTA interacts with SMA- and MAD-related protein 4 (SMAD4) in a proteome-scale map of the human protein–protein interaction network [4]. In 2009, we demonstrated the role of TCTA in human osteoclastogenesis as described above [1]. In addition, we have demonstrated that peptide A from TCTA protein inhibits the proliferation of small lung cells [5,6] and human synovial fibroblast-like cells from patients with rheumatoid arthritis [7]. However, the function of TCTA has not been clarified.

In the current study, to clarify the role of TCTA protein in bone metabolism *in vivo*, we generated TCTA gene transgenic (Tg), both systemic and osteoclast specific, mice and conditional knock-out (KO) mice and analysed their bones. We hypothesized that, in transgenic mice with over-expressed TCTA protein, bone volume decreases with activated osteoclastogenesis (Hypothesis 1). We also hypothesized that, in conditional knockout mice with TCTA protein-deficient osteoclast, bone volume increase with inhibited osteoclastogenesis (Hypothesis 2). Our findings completely supported Hypothesis 1. Surprisingly, Hypothesis 2 was not supported because bone volume decreased despite inhibited osteoclastogenesis. According to these findings, we speculated that TCTA protein expressed on osteoclasts plays a role as a 'coupling factor' *in vivo*.

Methods

Tg mice of TCTA

In this study we used groups of 9 weeks old female C57BL/6J mouse. Tg overexpression in systemic (TCTA-Tg1) or osteoclast-specific (TCTA-Tg2) TCTA was generated. Vectors containing TCTA gene were constructed. Pronuclear injection was performed. Tg

overexpression in osteoclast-specific TCTA (TCTA-Tg2) was performed under the TRAP promoter. The expression of TCTA DNA in TCTA-Tg1 was confirmed by Southern blotting using DNA form tails.

We analysed 2 TCTA-Tg1 mice, 2 TCTA-Tg2 mice, and 3 wild type (WT) mice. Bone histomorphometry, CT scan, and peripheral quantitative computed tomography (pQCT) of cancellous, cortical, and total bone in the metaphysis and diaphysis of the femur were performed.

Using Tg mice and WT mice different from mice described above, $2.5x10^4$/well of PBMCs peripheral blood mononuclear cells (PBMCs) were cultured under 100 ng/ml M-CSF alone for 4 days and then with 100 ng/ml MCSF and 100 ng/ml RANKL for 5 days. The total areas of osteoclasts formed were measured after the osteoclasts were stained using TRAP staining.

TCTA conditional KO mice

In this study we used groups of 10 weeks old female C57BL/6J mice. Homozygous TCTA KO under the TRAP gene promoter was generated as TCTA KO mice genetically deficient in osteoclasts. We analyzed 3 TCTA-KO mice and 3 WT mice. Bone analysis was performed as described above for TCTA-transgenic mice. Tg mice and KO mice were generated by Unitech Co., Ltd. (Kashiwa, Chiba prefecture, Japan). Bone analysis was performed at Kureha Corporation, Pharmaceuticals & Agrochemicals Div. (Shinjuku, Tokyo, Japan).

Statistical analysis

Data were analysed using Mann-Whitney U test (StatView®; Abacus Concepts Inc. Berkeley, CA). Data are presented as the mean ± s.d. Significant difference was defined as $P<0.05$.

Results

TCTA-transgenic (Tg) mice. Tg1, systemic transgenic; Tg2, osteoclast-specific transgenic

Bone histomorphometry was performed in secondary cancellous bone areas of Tg mice

There was a tendency for the number of osteoclasts [Oc.N/B.Pm (/100 mm)] to be higher in Tg1 mice than in WT mice or Tg2 mice (Figure 1A). Osteoid volume 7 (OV/BV%) was significantly higher in Tg mice including both Tg1 mice and Tg2 mice than in WT mice (Figure 1B, p=0.0039). There was no tendency in osteoblast surfaces (Ob.S/BS%) among WT mice, Tg1 mice, and Tg2 mice (Figure 1C). Trabecular numbers [Tb.N (/mm)] (Figure 1D, p=0.0339) and bone volume (BV/TV%) (Figure 1E, p=0.0339) were significantly lower in Tg mice including both Tg1 mice and Tg2 mice than in WT mice.

Micro-CT

There was a tendency for bone volumes in micro-CT to be lower in TCTA-Tg mice than in WT (Figure 2). Representative data are shown in Figure 2.

pQCT

pQCT of cancellous bone in the metaphysis of the femur was measured. pQCT values were significantly lower in Tg mice including both Tg1 mice and Tg2 mice than WT mice (Figure 3, p=0.0339).

Osteoclastogenesis using PBMCs of mice

There was a tendency for total areas of osteoclasts formed using PBMCs of mice to be higher in Tg1 mice than in WT mice or Tg2 mice (Figure 4).

TCTA-conditional KO mice

Bone histomorphometry in secondary cancellous bone area

There was a tendency for both osteoclast surfaces (Oc.S/BS %) and osteoclast numbers [Oc.N/B.Pm (/100 mm)] to be lower in KO mice than in WT mice (Figure 5A). There were tendencies for osteoid volumes (OV/BV%), osteoid surfaces (OS/BS%), and osteoid thickness (O.Th μm) to be lower in KO mice than in WT mice (Figure 5B). There was a tendency for osteoblast surfaces (Ob.S/BS%) to be lower in KO mice than in WT mice (Figure 5C). Trabecular thicknesses (Tb.Th μm) showed no differences between WT mice and KO mice (Figure 5D). In contrast, trabecular numbers [Tb.N (/mm)] were significantly lower in KO mice than in WT mice (Figure 5D, p=0.0495). In addition, trabecular separations (Tb.Sp micro m) were significantly higher in KO mice than in WT mice (Figure 5D, p=0.0495). Surprisingly, bone volumes (BV/TV %) were significantly lower in KO mice than in WT mice (Figure 5E, p=0.0495). In addition, bone volume of KO mice was about 60% of that of WT mice.

Micro-CT

It was difficult to detect significant differences between WT mice and KO mice on micro-CT (Figure 6).

pQCT

In the metaphysis, pQCT of cancellous bone of femur was significantly lower in KO mice than in WT mice (Figure 7A, p=0.0495), although no significant 9 differences were detected in pQCT of total bone and cortical bone between WT mice and KO mice (Figure 7A). In contrast, in the diaphysis, no significant differences were detected in total bone, cancellous bone or cortical bone between WT mice and WT mice (Figure 7B).

Discussion

In the current study, to clarify the role of TCTA protein *in vivo*, we first generated TCTA gene transgenic mice and analyzed their bones. We hypothesized that, in transgenic mice that over-express TCTA protein, bone volume would decrease with activated osteoclastogenesis (Hypothesis 1). There was a tendency for the number of osteoclasts to be higher in Tg1 mice than in WT mice or Tg2 mice (Figure 1A).

Trabecular numbers (Figure 1D) and bone volume (Figure 1E) was significantly lower in Tg mice than in WT mice, respectively. pQCT of the metaphysis of the femur was performed. pQCT values were significantly lower in Tg mice than in WT mice (Figure 3, p=0.0339). Thus, our findings completely supported Hypothesis 1, showing that TCTA protein plays an important role in osteoclastogenesis in TCTA gene Tg mice.

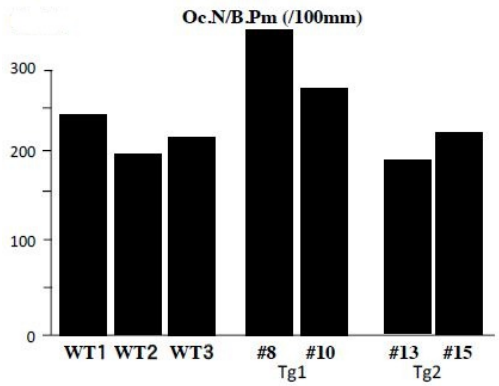

Figure 1A: Bone histomorphometry in secondary cancellous bone area of Tg mice (Tg1: systemic transgenic; Tg2: osteoclast-specific transgenic). Osteoclast number [Oc.N/B.Pm (/100 mm)].

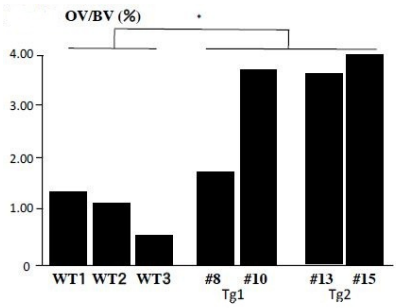

Figure 1B: Bone histomorphometry in secondary cancellous bone area of Tg mice (Tg1: systemic transgenic; Tg2: osteoclast-specific transgenic). Osteoid volume (OV/BV%) (**Note:** *p=0.0339).

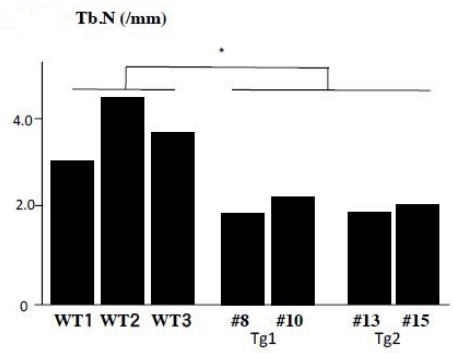

Figure 1C: Bone histomorphometry in secondary cancellous bone area of Tg mice (Tg1: systemic transgenic; Tg2: osteoclast-specific transgenic). Osteoblast surfaces (Ob.S/BS%).

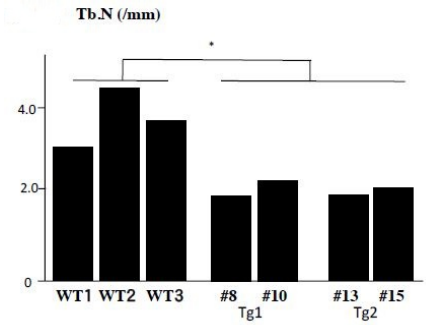

Figure 1D: Bone histomorphometry in secondary cancellous bone area of Tg mice (Tg1: systemic transgenic; Tg2: osteoclast-specific transgenic). Trabecular numbers [Tb.N (/mm)] (**Note:** *p=0.0339).

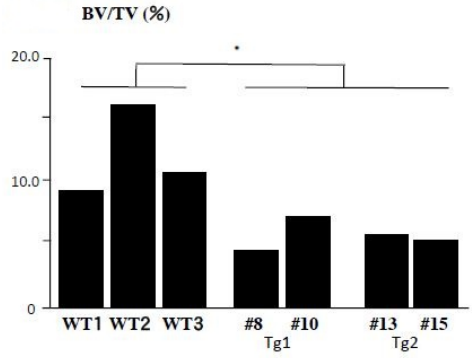

Figure 1E: Bone histomorphometry in secondary cancellous bone area of Tg mice (Tg1: systemic transgenic; Tg2: osteoclast-specific transgenic). Bone volume (BV/TV%) (**Note:** *p=0.0339).

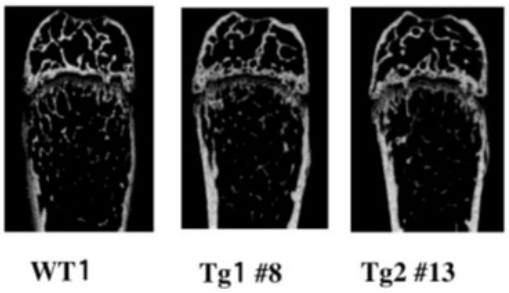

Figure 2: Micro-CT of WT, Tg1 and Tg2 mice (**Note:** Representative data are shown).

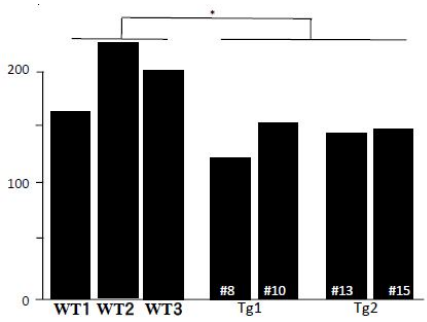

Figure 3: pQCT of cancellous bone of metaphysis of femur (mg/cm^3) (**Note:** *p=0.0339).

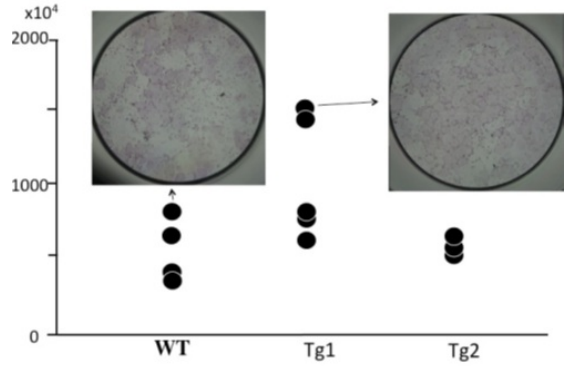

Figure 4: Total areas (μm^2) of osteoclasts formed using PBMCs (**Note:** Each dot shows the mean area of osteoclasts formed in triplicate experiments using PBMCs from each mouse).

We secondarily generated conditional knockout mice and analyzed their bones. We hypothesized that, in conditional knockout mice with TCTA protein-deficient osteoclast, bone volume would increase with inhibited osteoclastogenesis (Hypothesis 2). There was a tendency for both osteoclast surfaces and osteoclast numbers to be lower in KO mice than in WT mice (Figure 5A). However, trabecular numbers were significantly lower in KO mice than in WT mice (Figure 5D). In addition, trabecular separations (Tb.Sp) were significantly higher in KO mice than WT mice (Figure 5D).

Surprisingly, bone volumes were significantly lower in KO mice than in WT mice showing that the bone volume of KO mice was 60% of that of WT mice (Figure 5E). In addition, in the metaphysis, the pQCT of cancellous bone was significantly lower in KO mice than in WT mice (Figure 7A, p=0.0495).

Thus, Hypothesis 2 was not supported because bone volume decreased despite inhibited osteoclastogenesis. According to these findings, we considered the possibility of an effect of TCTA protein not only on the differentiation and function of osteoclasts but also on those of osteoblasts (Figure 8).

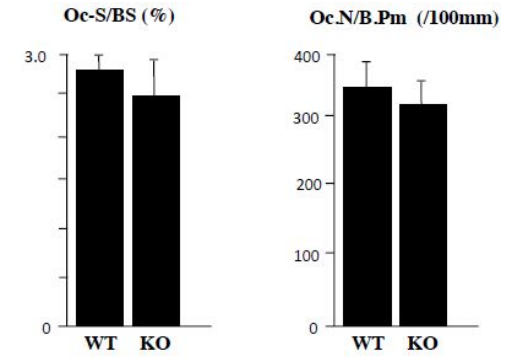

Figure 5A: Bone histomorphometry in secondary cancellous bone area of KO mice. Osteoclast surfaces (Oc.S/BS%) and Osteoclast number [Oc.N/B.Pm (/100 mm)].

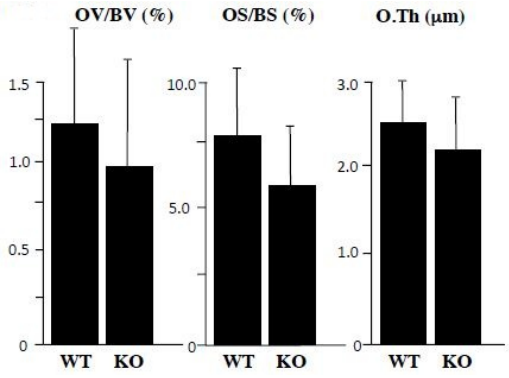

Figure 5B: Bone histomorphometry in secondary cancellous bone area of KO mice. Osteoid volumes (OV/BV%), Osteoid surfaces (OS/BS%), and Osteoid thickness (O.Th μm).

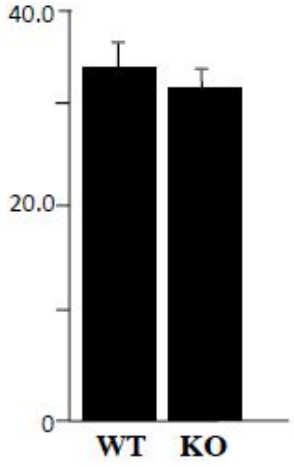

Figure 5C: Bone histomorphometry in secondary cancellous bone area of KO mice. Osteoblast surfaces (Ob.S/BS%).

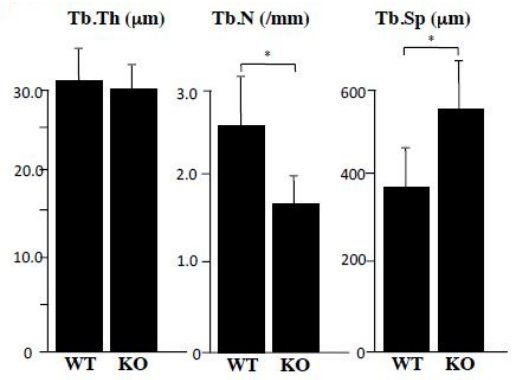

Figure 5D: Bone histomorphometry in secondary cancellous bone area of KO mice. Trabecular thicknesses (Tb.Th μm), Trabecular numbers [Tb.N (/mm)] and Trabecular separations (Tb.Sp μm) (**Note:** *p=0.0495).

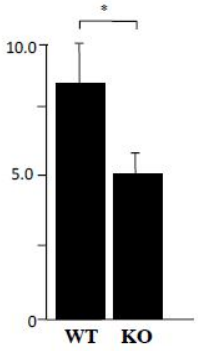

Figure 5E: Bone histomorphometry in secondary cancellous bone area of KO mice. Bone volume (BV/TV%) (**Note:** *p=0.0495).

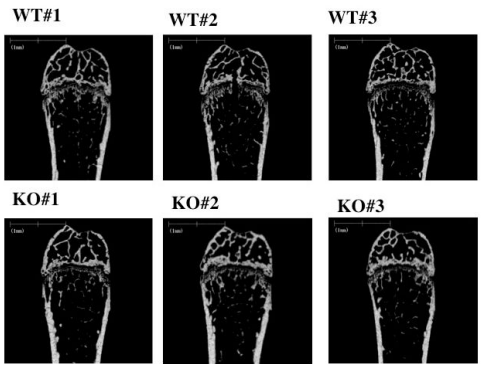

Figure 6: Micro-CT of WT and KO mice (**Note:** Representative data are shown).

The mechanism by which TCTA protein functions on osteoblasts is not clear. Our findings in the conditional KO mice suggest that the differentiation or function of osteoblasts requires the expression of TCTA on osteoclasts. In the previous study, we speculated that both TCTA protein and a putative counterpart to TCTA protein play roles in the fusion process of osteoclastogenesis [1].

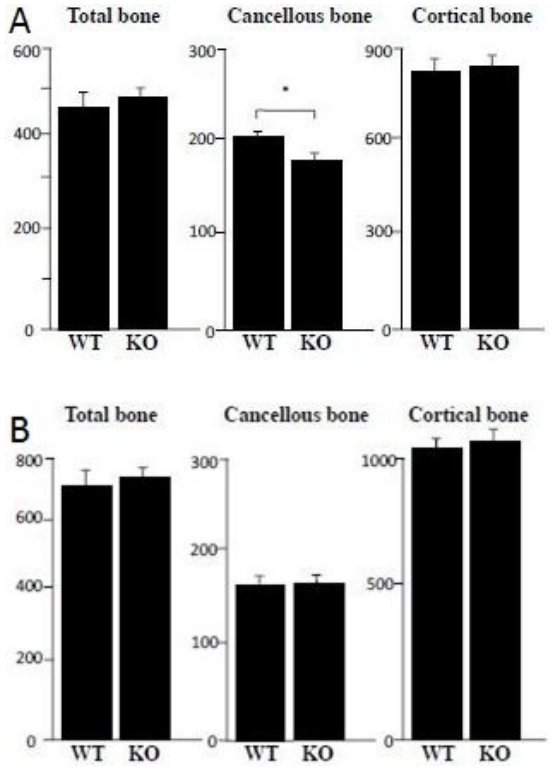

Figure 7: pQCT of total bone, cancellous bone and cortical bone of Metaphysis (A) or Diaphysis (B) of femur (mg/cm^3) (**Note:** *p=0.0495).

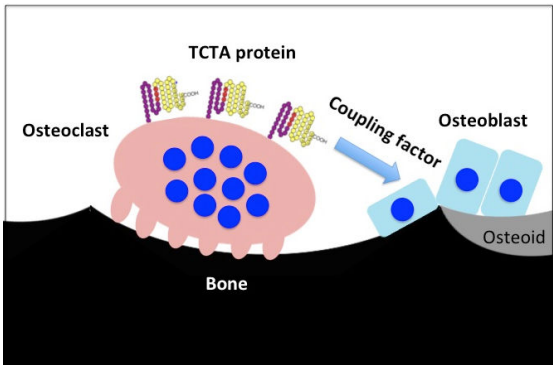

Figure 8: TCTA protein as a novel coupling factor (**Note:** The structure of TCTA protein) [1].

It is possible that the counterpart of TCTA protein is expressed on osteoblasts. We also speculated that peptides derived from TCTA protein may play a role in the differentiation or function of osteoblasts. Thus, we speculate that TCTA is a novel "coupling factors" by which osteoclasts stimulate osteoblasts directly or directly, such as epherin B2, sphingosine 1-phospate, transforming growth factor (TGF)-β,

insulin-like growth factor (IGF)-1, and collagen triple helix repeat containing (CTHRC)1, in the bone metabolism [8-12] (Figure 8). We are now trying to identify the counterpart of TCTA protein. In addition, we are also investigating the function of TCTA protein in the differentiation or function of osteoblasts *in vitro*.

Osteoclastogenesis *in vitro* was analysed using PBMCs from Tg1 mice, Tg2 mice, and WT mice (Figure 4). There was a tendency for the total areas of osteoclasts formed using PBMCs of mice to be higher in Tg1 mice than in WT mice or Tg2 mice (Figure 4). In Tg1 mice, TCTA protein was over-expressed in monocytes as the progenitor of osteoclasts. In contrast, in Tg2 mice, TCTA protein was over-expressed in only osteoclasts expressing TRAP. In the previous study, we demonstrated the role that TCTA protein plays in the fusion process of osteoclastogenesis [1]. According to these findings, we speculate that TCTA proteins expressed on monocytes are more important than those expressed on osteoclasts in the process of osteoclastogenesis *in vitro*. This speculation is also supported *in vivo*, because there was a tendency for the osteoclast number to be higher in Tg1 mice than in WT mice or Tg2 mice (Figure 1A).

In conclusion, we have demonstrated that TCTA protein expressed on osteoclast plays a role as a novel 'coupling factor' *in vivo*. We are now investigating the function of TCTA protein in the differentiation or function of osteoblasts *in vitro*.

Acknowledgement

We thank Ms. Hanae Kikuchi (Tokyo Women's Med. Univ.) for her valuable technical assistance.

Declaration

Authors declare that there is no conflict of interest regarding the publication of this paper.

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