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Osteoporotic bone formation in mice lacking *tob2*; involvement of Tob2 in RANK ligand expression and osteoclasts differentiation

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Abstract Mice lacking *tob2*, a member of the antiproliferative family genes, had decreased bone mass, and the number of osteoclasts differentiated from bone marrow cells was increased. Overexpression of Tob2 in stromal cells repressed vitamin D_3-induced osteoclasts formation. Furthermore, expression of *RANKL* mRNA in stromal cells was increased in the absence of Tob2 and decreased in the presence of Tob2. Tob2 interacted with vitamin D_3 receptor (VDR), which suggests its involvement in vitamin D_3 receptor-mediated regulation of transcription. Because VDR regulates *RANKL* expression, our data suggest that Tob2 negatively regulates formation of osteoclasts by suppressing *RANKL* expression through its interaction with VDR.

Keywords: Bone mass; Osteoclast; Gene targeting; *tob* Family; Vitamin D_3

1. Introduction

Tob2 is a member of Tob/BTG family of antiproliferative proteins, and exogenous expression of Tob family proteins suppresses growth of NIH3T3 cells by inhibiting G1 progression of the cell cycle [1]. Tob2, like other Tob family members, interacts with Caf1/Cnot7 [2], which is a component of the CCR4-NOT protein complex [3]. Caf1/Cnot7 interacts with nuclear receptors ERα and RXRβ [4,5]. Moreover, the CCR4-NOT complex participates in control of expression of specific sets of genes [3]. Both BTG1 and BTG2 associate with HoxB9 and enhance HoxB9-mediated transcription [6]. Tob associates with the Smads transcription complex and affects Smad-mediated gene expression [7,8]. These findings suggest that Tob family proteins are involved in transcriptional regulation. Because Tob family members do not have DNA-binding domains, they are thought to function as either co-activators or co-repressors in conjunction with various transcription factors.

We reported previously that Tob is involved in bone metabolism and that *tob*-deficient mice develop an osteopetrotic phenotype [7,9]. The high level of bone mass seen in adult *tob*-deficient mice was due to an increase in bone formation by osteoblasts with little alteration in bone resorption. We report here that, in contrast to *tob*-deficient mice, adult mice lacking *tob2* show low levels of bone mass due to an increase in the number of osteoclasts.

2. Materials and methods

2.1. Generation of $tob2^{-/-}$ mice

An 8-kbp and a 1.5-kbp fragment flanking the 5′ and 3′ sites, respectively, of an exon of the *tob2* gene [10] were ligated to the 5′ end of the *nlsLacZ* and the 3′ end of the *NeoR* genes of pBluescript SK+ (Stratagene), respectively. The *Diphtheria toxin A* (DTA) gene was ligated to the 3′ end of the targeting cassette. The plasmid was linearized and electroporated into J1 embryonic stem (ES) cells [11]. After selecting G418-resistant ES clones, *tob2*-targeted clones were identified by Southern blot hybridization using probe 1 (Fig. 1A). The cells carrying the targeted allele were injected into C57BL/6J blastocysts. Homozygosity of the *tob2* knockout allele was confirmed by Southern blotting and immunoblotting as described [5]. Anti-Tob2 antibodies used were described [2].

2.2. Microfocus-X-ray computed tomography (μCT) analysis of bone and bone histomorphometry

To measure bone volume, femora of 9-month-old male mice were subjected to μCT analysis as described previously [9]. For bone histomorphometry, femora of 9-month-old male mice were analyzed as described previously [7].

2.3. Retroviral transduction

Mouse *tob2* coding region (*mtob2*) and flag-tagged human *tob2* cDNA (*htob2F*) were inserted between the LTR sequences of pMX-puro expression plasmid, and the resulting plasmids were transfected into Plat E cells for retrovirus production as described [12].

2.4. Cell culture

COS-7 monkey kidney-derived cells and ST2 mouse bone marrow-derived stromal cells were cultured in DMEM and αMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (Sigma), respectively. For real-time reverse-transcriptase polymerase chain reaction (RT-PCR), ST2 was cultured in phenol red-free αMEM supplemented with 10% charcoal-stripped FBS, and treated with indicated concentrations

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Abbreviations: RANKL, receptor activator of NF-κB ligand; FBS, fetal bovine serum; RT-PCR, reverse-transcriptase polymerase chain reaction; MEFs, mouse embryonic fibroblasts; TRAP, tartrate-resistant acid phosphatase; GST, glutathione-s-transferase

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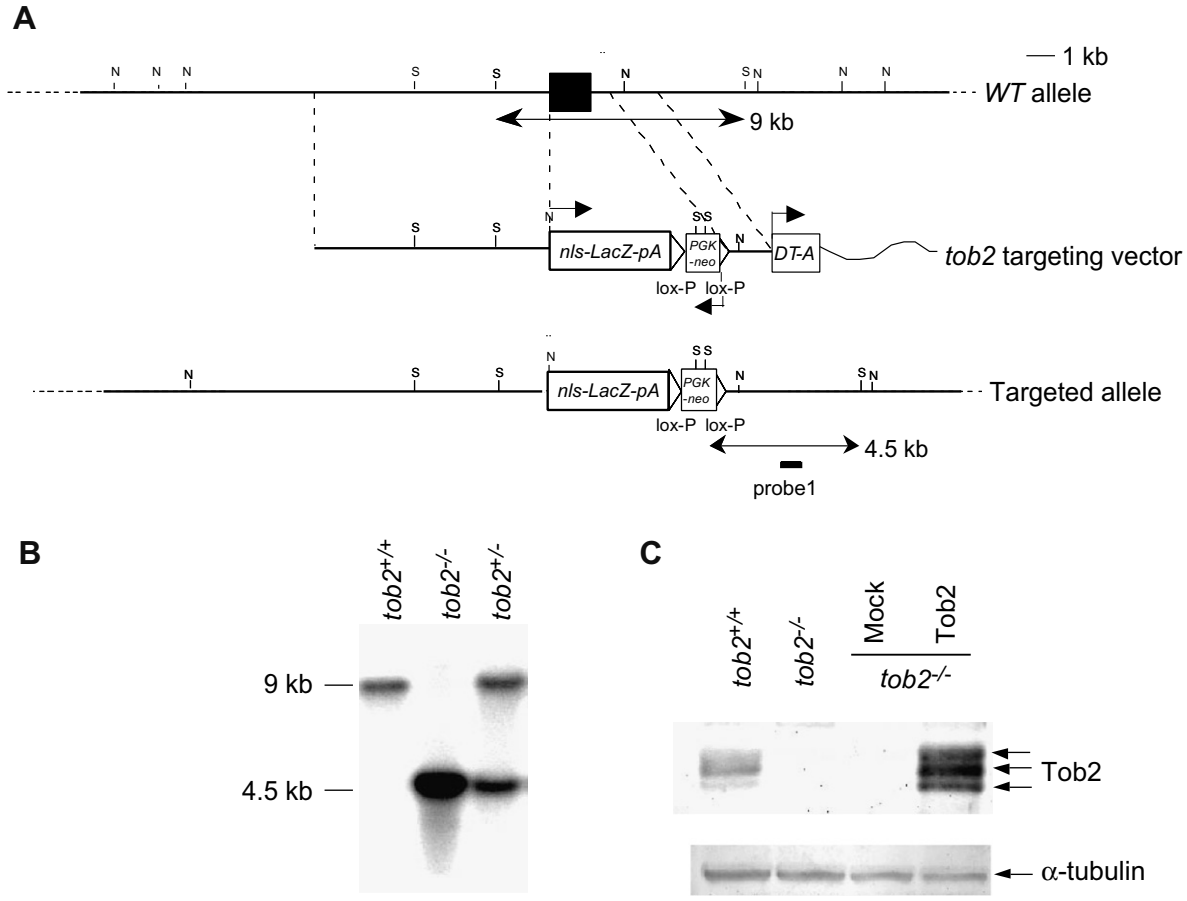

Fig. 1. Targeted disruption of the *tob2* gene. (A) Schematic illustration of *tob2* targeting vector. The coding region is shown as a closed box. Arrows on each box show the direction of each gene cassette. The SphI-digested DNA fragment detected by probe1 is shown as two-headed arrows. S, SphI; N, NcoI. (B) Tail DNAs from F1 progeny of two heterozygote intercrosses were digested with SphI and subjected to Southern blot hybridization with probe 1. Sizes of the DNA fragments are indicated on the left. (C) Protein lysates prepared from primary embryonic fibroblasts infected with or without *mtob2* expression viruses were analyzed by immunoblotting with the anti-Tob2 antibodies (top panel) and anti-α-tubulin antibodies (bottom panel).

of 1,25-$(OH)_2D_3$, 10 nM dexamethasone and 1 μM prostaglandin E_2. Bone marrow cells were collected from femur and cultured in αMEM supplemented with 10% FBS. The cultured medium was changed every 2 days for one week. Mouse embryonic fibroblasts (MEFs) were obtained from 14.5-day-old embryos and cultured in DMEM containing 10% FBS and 50 μM β-mercaptoethanol.

2.5. In vitro osteoclast-like cell formation

Mouse splenocytes or bone marrow cells (10^6/well) were cultured for 7 days in the presence of 10 ng/ml M-CSF and 30 or 100 ng/ml soluble form of receptor activator of NF-κB ligand (sRANKL) or co-cultured with ST2 cells (2×10^4/well) in the presence of 10 nM 1,25-$(OH)_2D_3$ and 1 μM prostaglandin E_2 in 24 well plates. Mouse bone marrow cells (10^6/well) were cultured for 7 days in the presence of 10 nM 1,25-$(OH)_2D_3$ and 1 μM prostaglandin E_2 in 24-well plates. The cells were then stained for tartrate-resistant acid phosphatase (TRAP), and the number of osteoclast-like TRAP-positive multinucleated (>3 nuclei) cells was counted.

2.6. Real-time RT-PCR

Total RNAs were reverse transcribed by a SuperScriptII First-Strand Synthesis System (Invitrogen) using oligo(dT). Real-time RT-PCR was performed as described [13]. Primer sequences were as follows: Tob2 forward 5′-GCATTCATGCCTGTGAGTGTGTGGC TTGC-3′, Tob2 reverse 5′-GGGTTTCTCGGGCTTAGCCTCGTGT CA-3′, RANKL forward 5′-ATTTGCACACCTCACCATCAA-3′, RANKL reverse 5′- TAGAGATCTTGGCCCAGCCTC-3′, HPRT forward 5′-GTAATGATCAGTCAACGGGGGAC-3′, and HPRT reverse 5′-CCAGCAAGCTTGCAACCTTAACCA-3′.

2.7. Glutathione-s-transferase (GST) pull-down

Cells were lysed with TNE buffer 36 h after transfection and subjected to affinity pull-down assay as described [5]. Anti-c-Myc and anti-GST antibodies were from Santa Cruz Biotechnology.

3. Results

3.1. Bone mass is decreased in $tob2^{-/-}$ mice

The *tob2*-targeting vector (Fig. 1A) was introduced into J1 ES cells to obtain homologous recombinants. Among 96 independently selected ES cell clones, two *tob2*-targeted clones were injected into C57BL/6 recipient blastocysts, and male chimeras were crossed to C57BL/6 females. Germ-line transmission of the ES cell genome was monitored by inspection for agouti coat color and by Southern blot hybridization. $tob2^{-/-}$ mice were obtained by crossing $tob2^{+/-}$ mice (Fig. 1B and C). $tob2^{-/-}$ mice were alive at birth and were obtained at predicted Mendelian frequencies. No apparent phenotypic abnormalities were observed within a year after birth except that the bone mass was decreased compared with that of $tob2^{+/+}$ littermates. μCT analysis revealed that the bone mass of $tob2^{-/-}$ mice was approximately 62% that of $tob2^{+/+}$ littermates at 9 months of age (Fig. 2A and B). Further histomorphometric analysis

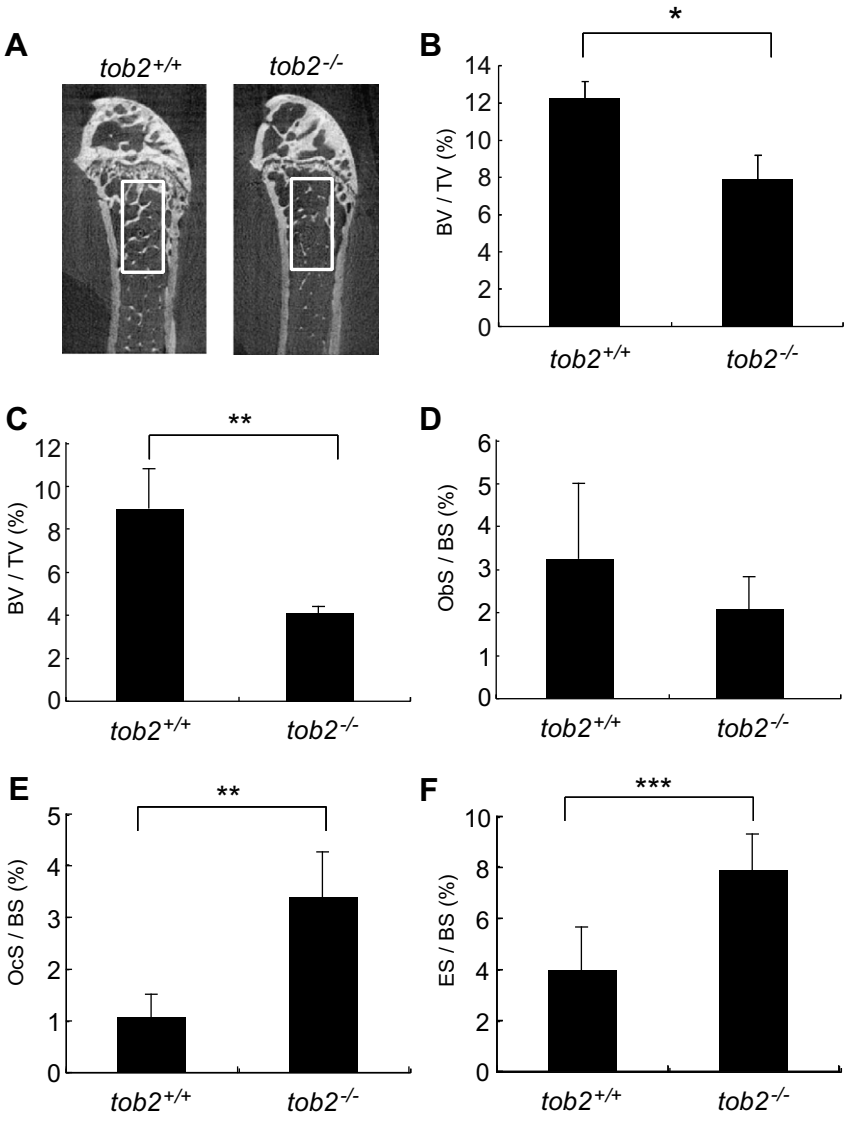

Fig. 2. *tob2* deficiency reduces bone volume. (A) μCT analysis of femora of $tob2^{+/+}$ or $tob2^{-/-}$ mice. (B) μCT-based quantification of the bone volume (BV)/total tissue volume (TV) within the areas indicated by the rectangles in (A). (C–H) Histomorphometric analysis of markers of $tob2^{+/+}$ or $tob2^{-/-}$ mice. (C) Bone to tissue ratio (in volume) (BV/TV) reflects bone mass. (D, E) Osteoblast surface (ObS/BS) and osteoclast surface (OcS/BS) indicate the portion of bone surface covered by osteoblasts and osteoclasts, respectively. (F) Eroded surface (ES/BS) represents the function of osteoclasts. Bars represent means ± S.D. Asterisks indicate a statistically significant difference between $tob2^{+/+}$ and $tob2^{-/-}$ mice ($^{*}P < 0.01$, $^{**}P < 0.025$, $^{***}P < 0.05$ by Student's *t* test, $n = 3$, $tob2^{+/+}$; 4, $tob2^{-/-}$ for A and B, $n = 3$ each for D–F).

showed that the bone volume/total tissue volume (BV/TV), a histomorphometric index of trabecular bone mass, was decreased in $tob2^{-/-}$ mice as compared with that of wild-type littermates (Fig. 2C). To determine whether the increase in trabecular bone mass was due to altered bone remodeling, we examined three markers: osteoblast surface (ObS/BS, proportion of bone surface covered with osteoblasts), osteoclasts surface (OcS/BS, proportion of bone surface covered with osteoclasts) and eroded surface (ES/BS, proporpositive of bone surface eroded by osteoclasts) in $tob2^{+/+}$ and $tob2^{-/-}$ mice. The osteoblast surface in $tob2^{-/-}$ mice has no statistically significant difference with that in $tob2^{+/+}$ mice (Fig. 2D), suggesting that decreased bone mass in $tob2^{-/-}$ mice was not due to reduced osteoblast numbers. In contrast, the osteoclast surface and eroded surface, which are reliable indicators of osteoclast population and function, were increased significantly in $tob2^{-/-}$ mice and was 317% and 199% greater than that of $tob2^{+/+}$, respectively (Fig. 2E and F). Thus, we concluded that the decreased bone mass in $tob2^{-/-}$ mice was due to increased osteoclast numbers and accelation of the bone resorption rate.

3.2. Tob2 expression in bone forming and bone modifying cells

Tob2 is ubiquitously expressed throughout most of tissues [2,10]. The ubiquitous expression was confirmed by LacZ staining using tissues that obtained from $tob2^{+/-}$ mice (data not shown). To address whether Tob2 is expressed in cells related to bone formation, we examined expression of Tob2 in stromal cells and osteoclasts. Tob2 was expressed in both stromal cells derived from bone marrow and stromal cell line ST-2,

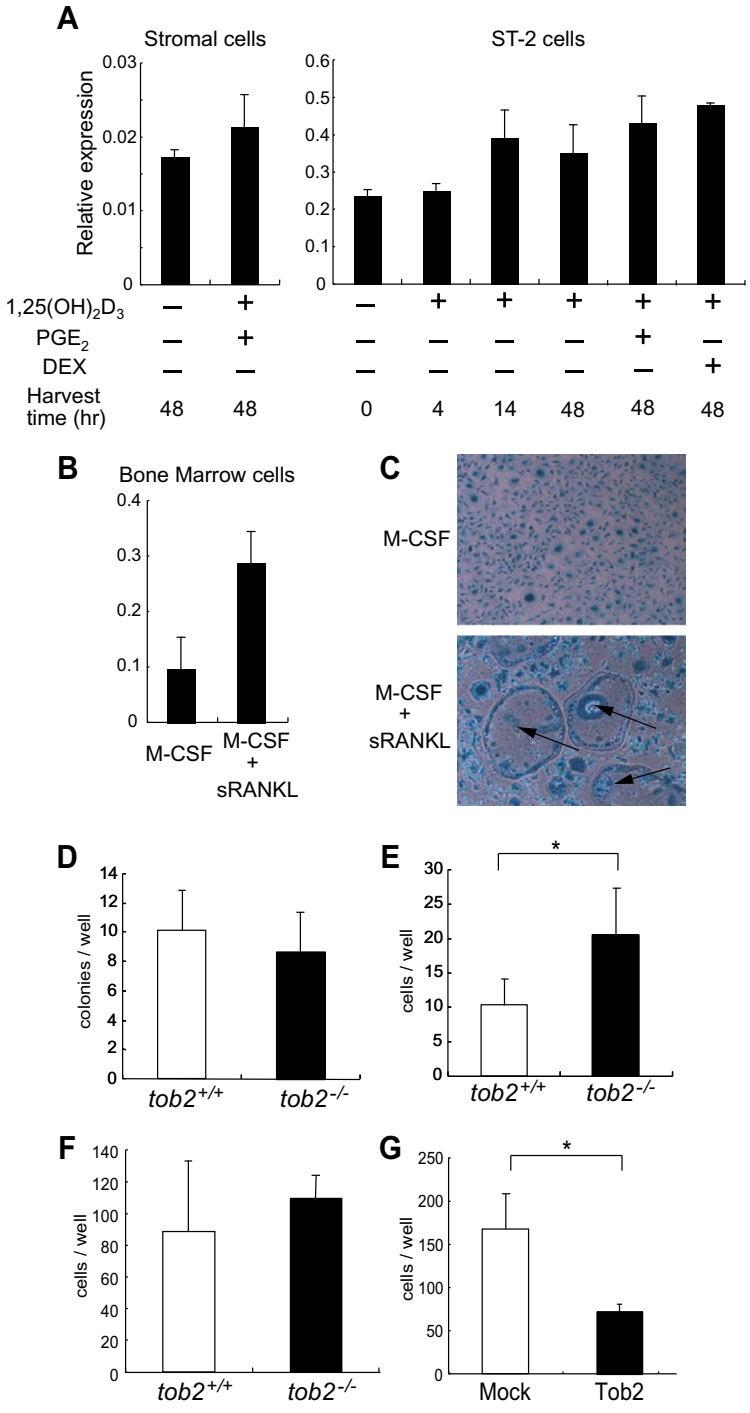

Fig. 3. Effect of Tob2 on osteoclast/osteoblast formation. (A) Stromal cells were collected from wild type mice bone marrow and stimulated with or without 10 nM 1,25-$(OH)_2D_3$ and 1 μM prostaglandin E_2. Total RNA was collected 48 h after stimulation. ST-2 cells were stimulated with 10 nM 1,25-$(OH)_2D_3$, 10 nM dexamethasone (DEX) and 1 μM prostaglandin E_2 (PGE_2), then total RNA was collected at indicated time points and subjected to semi-quantitative real-time RT-PCR analysis to examine Tob2 expression. Relative expression levels that compared with HPRT are shown. (B) Bone marrow cells were cultured with 10 ng/ml M-CSF or 10 ng/ml M-CSF and 100 ng/ml sRANKL. Total RNA was collected 7 days after stimulation and subjected to semi-quantitative real-time RT-PCR analysis as in (A). Relative expression levels that compared with HPRT are shown. (C) Tob2 expression reported by LacZ staining is shown. Cells are cultured as in (B) and stained with X-gal. All the cells including stromal cells, osteoclast precursors (in field of M-CSF culture) and osteoclasts (in field of M-CSF+sRANKL culture; indicated with arrows) stained with X-gal. (D) In vitro osteoblast-colony-formation assay with $tob2^{+/+}$ or $tob2^{-/-}$ bone marrow cells. (E) In vitro osteoclast-formation assay with $tob2^{+/+}$ or $tob2^{-/-}$ bone marrow cells cultured with 10 nM 1,25-$(OH)_2D_3$ and 1 μM prostaglandin E_2. (F) In vitro osteoclast-formation assay with $tob2^{+/+}$ or $tob2^{-/-}$ splenocytes cultured with 10 ng/ml M-CSF and 30 ng/ml sRANKL. (G) In vitro osteoclast-formation assay performed by co-culturing splenocytes with ST-2 cells infected with mock or *htob2F* expression viruses in the presence of 10 nM 1,25-$(OH)_2D_3$ and 1 μM prostaglandin E_2. Bars represent means ± S.D. * $p < 0.05$, by Student's t test, $n = 3$.

and the expression level was slightly increased when the cells stimulated with $1,25(OH)_2D_3$ (Fig. 3A). Furthermore, consistent with previous report [7], Tob2 expression was higher in osteoclasts that were differentiated from bone marrow cells upon M-CSF and sRANKL stimulation than in bone marrow cells cultured in the presence of M-CSF which contains stromal cells and osteoclast precursor cells (Fig. 3B). LacZ staining of each cell population confirmed Tob2 expression in both cell types (Fig. 3C).

3.3. Tob2 decreases osteoclast differentiation

To examine regulation of osteoblasts or osteoclasts formation of *tob2*$^{-/-}$ mice, we cultured bone marrow cells from femur and performed differentiation assays. The number of osteoblast colonies differentiated from bone marrow cells of *tob2*$^{+/+}$ and *tob2*$^{-/-}$ mice did not differ significantly (Fig. 3D). In contrast, the number of osteoclasts differentiated from bone marrow cells of *tob2*$^{-/-}$ mice upon stimulation with osteotropic factor, $1,25(OH)_2D_3$, was significantly higher than that from *tob2*$^{+/+}$ mice (Fig. 3E). These results are consistent with the results shown in vivo (Fig. 2).

RANKL produced by stromal cells activates RANK expressed on the surface of osteoclast precursors, resulting in differentiation, formation, fusion and survival of osteoclasts. To investigate the ability of osteoclast precursors in *tob2*$^{-/-}$ mice to differentiate into mature osteoclasts, we cultured splenocytes containing osteoclast precursors from *tob2*$^{+/+}$ and *tob2*$^{-/-}$ mice with sRANKL. We found that the rates of osteoclast formation from osteoclast precursors did not differ significantly between *tob2*$^{-/-}$ and *tob2*$^{+/+}$ mice (Fig. 3F). These data suggest that RANK expression is not altered in the absence of Tob2. We then examined the effect of Tob2 on the function of stromal cells. We found that differentiation of splenocytes into osteoclasts was clearly suppressed when co-cultured with Tob2-expressing ST2 cells (Fig. 3G). On the basis of these data, we concluded that Tob2 negatively regulates stromal cell-induced differentiation of osteoclasts.

3.4. Tob2 regulates RANKL expression in stromal cells

RANKL activates mature osteoclasts, inducing actin ring formation and bone resorption. Osteoprotegerin (OPG) secreted by stromal cells acts as a soluble neutralizing receptor for RANKL. The balance of these two proteins affects bone mass. Therefore, we examined expression of RANKL and OPG in *tob2*-deficient or -overexpressing stromal cells. While expression of OPG did not correlate clearly with expression of Tob2 (data not shown), RANKL expression induced by $1,25(OH)_2D_3$ and prostaglandin E_2 was increased in *tob2*$^{-/-}$ stromal cells, and reduced in Tob2-overexpressing ST-2 stromal cells (Fig. 4A and B). These data suggest that Tob2 is involved in regulation of expression of RANKL. Because $1,25(OH)_2D_3$-induced RANKL expression is regulated through VDR [14], we speculated that Tob2 may functionally and/or physically interact with VDR. To examine this possibility, GST-tagged Tob2 and myc-tagged VDR were expressed in COS-7 cells. Cell lysates were then subjected to GST pull-down assay. The results revealed that Tob2 interacts with VDR regardless of $1,25(OH)_2D_3$ stimulation (Fig. 5).

4. Discussion

Osteoclasts are multinucleated giant cells that are present specifically in bone and that adsorb bone. Osteoclasts differentiate from hematopoietic cells, and osteoblasts and/or stromal cells are crucial for osteoclast development [15]. Bone marrow stromal cells express an osteoclast differentiation factor called RANKL. Treatment of hematopoietic cells with a combination of M-CSF and the soluble form of RANKL induces osteoclast differentiation in vitro [16]. RANKL and its receptor, RANK, are essential for osteoclast differentiation [17], and targeted disruption of RANKL or RANK induces osteopetrosis in mice [18,19]. Overexpression of RANKL leads to osteoporotic skeletal changes [20]. These results clearly indicate that the RANKL/RANK pathway is essential for osteoclast development in vivo.

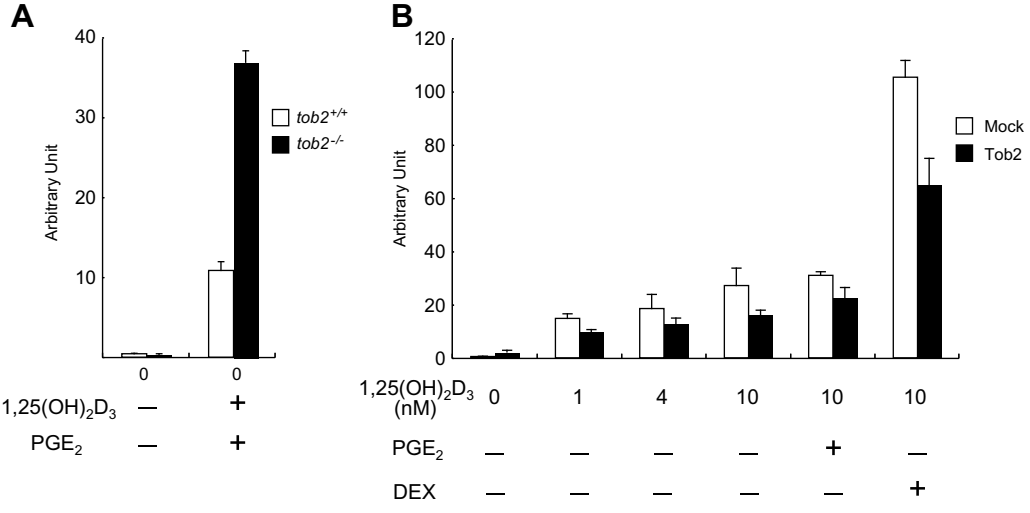

Fig. 4. Effect of Tob2 on RANKL expression. (A) Stromal cells were collected from *tob2*$^{+/+}$ or *tob2*$^{-/-}$ bone marrow and stimulated with or without 10 nM $1,25\text{-}(OH)_2D_3$ and 1 μM prostaglandin E_2. Total RNA was collected 48 h after stimulation and subjected to semi-quantitative real-time RT-PCR analysis. Expression of RANKL was normalized to that of HPRT. Bars represent means ± SD. (B) Semi-quantitative real-time RT-PCR analysis of RANKL expression in ST2 stromal cell lines infected with mock or *htob2F* expression virus. Cells were stimulated with 0, 1, 4, 10 nM $1,25\text{-}(OH)_2D_3$, 10 nM dexamethasone (DEX) and 1 μM prostaglandin E_2 (PGE_2), then analyzed as in (A). ST2 stromal cell lines infected *mtob2* expression virus also showed similar results (data not shown).

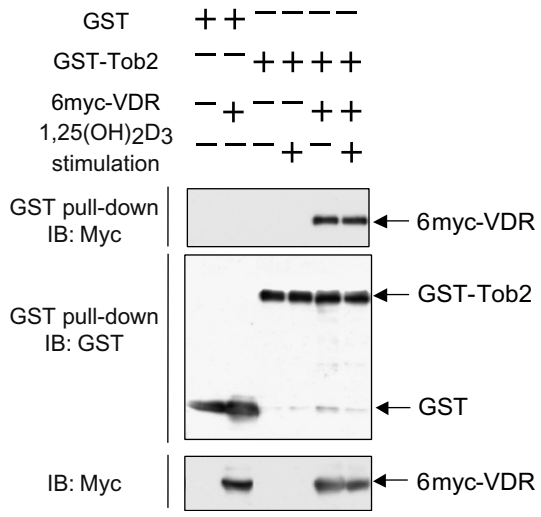

Fig. 5. Tob2 interacts with VDR. GST or GST-fused Tob2 was coexpressed with 6Myc-tagged VDR in COS-7 cells and stimulated with or without 1,25-$(OH)_2D_3$. Total protein extracts were subjected to GST-pull-down assay followed by immunoblotting (IB) with indicated antibodies. The bottom panel shows expression of 6Myc-tagged VDR.

In the present study, we found that *tob2*-deficient mice have an osteoporotic phenotype, due most likely to an increased number of osteoclasts (Figs. 2E, F and 3E). This is in contrast with *tob*-deficient mice, which show osteopetrotic changes because of increased proliferation and differentiation of osteoblasts [7]. Because expression of Tob2 was shown in both stromal cells and osteoclasts (Fig. 3A–C), Tob2 in stromal cells or osteoclasts might underlie the osteoporotic phenotype of *tob2*-deficient mice. Our data suggest that RANK expression in osteoclast lineage cells is normal in the absence of Tob2 (Fig. 3F). In contrast, expression of RANKL by stromal cells is increased in the absence of Tob2 (Fig. 4). The ability of stromal cells to induce osteoclast differentiation is suppressed by exogenously expressed Tob2 (Fig. 3G). Therefore, we tentatively concluded that Tob2 is involved in stromal cell-mediated osteoclast differentiation. Most likely Tob2 may negatively regulate RANKL expression in stromal cells. The function of Tob2 in osteoclasts remains to be clarified.

Tob/BTG family proteins are involved in transcriptional regulation. For example, BTG1-associated protein Caf1/Cnot7 interacts with estrogen receptor [4] as well as RXRβ [5]. We hypothesized that Tob2 might directly or indirectly regulate the transcription of the *RANKL* gene. Expression of *RANKL* is regulated by $1,25(OH)_2D_3$ and VDR as well as RXRβ [14]. Because Tob2 interacts physically with VDR (Fig. 5), Tob2 is likely involved in VDR-mediated transcriptional regulation of the *RANKL* gene. The precise mechanism by which RANKL expression is mediated by Tob2 and VDR remains unclear. Because Caf1/Cnot7 interacts with Tob2 and with RXRβ, Tob2 could be involved in regulation of the RANKL expression through an interaction with Caf1/Cnot7 as well.

Among the Tob/BTG family proteins, Tob is very similar to Tob2. Nevertheless, *tob*-deficient mice and *tob2*-deficient mice show opposite phenotypes in terms of bone formation. Examination of bone remodeling in *tob*/*tob2* double-knockout mice should further our understanding of bone formation.

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