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Dlx3 Plays a Role as a Positive Regulator of Osteoclast Differentiation

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Dlx3 is a homeodomain protein and is known to play a role in development and differentiation of many tissues. Deletion of four base pairs in *DLX3* (NT3198) is causally related to tricho-dento-osseous (TDO) syndrome (OMIM # 190320), a genetic disorder manifested by taurodontism, hair abnormalities, and increased bone density in the cranium. Although the observed defects of TDO syndrome involves bone, little is known about the role of Dlx3 in bone remodeling process. In this study, we examined the effect of wild type *DLX3* (wtDlx3) expression on osteoclast differentiation and compared it with that of 4-BP DEL *DLX3* (TDO mtDlx3). To examine whether Dlx3 is expressed during RANKL-induced osteoclast differentiation, RAW264.7 cells were cultured in the presence of receptor activator of nuclear factor-B ligand (RANKL). Dlx3 protein level increased slightly after RANKL treatment for 1 day and peaked when the fusion of prefusion osteoclasts actively progressed. When wtDlx3 and TDO mtDlx3 were overexpressed in RAW264.7 cells, they enhanced RANKL-induced osteoclastogenesis and the expression of osteoclast differentiation marker genes such as calcitonin receptor, vitronectin receptor and cathepsin K. Since osteoclast differentiation is critically regulated by the balance between RANKL and osteoprotegerin (OPG), we examined the effect of Dlx3 overexpression on expression of RANKL and OPG in C2C12 cells in the presence of bone morphogenetic protein 2. Overexpression of wtDlx3 enhanced RANKL mRNA expression while slightly suppressed OPG expression. However, TDO mtDlx3 did not exert significant effects. This result suggests that inability of TDO mtDlx3 to regulate expression of RANKL and OPG may contribute to increased bone density in TDO syndrome patients. Taken together, it is suggested that Dlx3 play a role as a positive regulator of osteoclast differentiation via up-regulation of osteoclast differentiation-associated genes in osteoclasts, as well as via increasing the ratio of RANKL to OPG in osteoblastic cells.

Key words : Dlx3, TDO mutant Dlx3, Osteoclast differentiation, RANKL, OPG

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INTRODUCTION

Bone mass is maintained by the balance between osteoclastic bone resorption and osteoblastic bone formation. Osteoclasts, fully differentiated multinucleated cells of a hemopoietic origin, are the primary bone-resorbing cells. The receptor activator of nuclear factor-B ligand (RANKL) is a membrane-bound protein produced by osteoblasts/bone marrow stromal cells and is known to be both sufficient and necessary for osteoclast formation *in vitro* in the presence of macrophage-colony stimulating factor (M-CSF, Lacey *et al.*, 1998; Yasuda *et al.*, 1998a; Quinn *et al.*, 1998). Osteoprotegerin (OPG) is also secreted from the osteoblasts/bone marrow stromal cells and inhibits osteoclastogenesis by acting as a decoy receptor of RANKL (Simonet *et al.*, 1998; Yasuda *et al.*, 1998b). Many osteotropic agents manifest their inhibitory or stimulatory effects on bone resorption via regulation of RANKL and OPG expression level (Suda *et al.*, 1999; Aubin and Bonnelye, 2000).

Homeodomain (HD) proteins comprise a large group of transcription factors that bind to TAAT sequence motif (McGinnis and Krumlauf, 1992). The Distal-less family (Dlx) is one of the HD protein families and has been suggested to play important roles in development and differentiation of many tissues (Morasso *et al.*, 1996; Anderson *et al.*, 1997). In vertebrates, there are six Dlx genes arranged in three clusters (Dlx1/Dlx2, Dlx3/Dlx4, Dlx5/Dlx6; Ghanem *et al.*,

2003). Among these Dlx members, considerable attention has been paid to Dlx3. Dlx3 is a 287 amino acid protein with the HD located between 130-189aa. Mutations in *DLX3* are believed to be causally related to tricho-dento-osseous (TDO) syndrome (OMIM #190320), an autosomal dominant genetic disorder manifested by taurodontism, hair abnormalities, and increased bone density in the cranium (Price *et al.*, 1998a, 1998b; Wright *et al.*, 1997). The genetic defect leading to TDO syndrome appears to be associated with a four nucleotide deletion just downstream of the HD (4-BP DEL, NT3198), resulting in frame shift and premature truncation of the DLX3 protein.

Although the observed defects of TDO syndrome involves bone as well as ectodermal derivatives, little is known about the role of Dlx3 in bone development and remodeling process. Recently, several studies have shown that Dlx3 is expressed in osteoblasts and that knock-down of Dlx3 by RNA interference causes blockade of mature osteoblast formation by suppressing bone related marker genes, indicating the critical role of Dlx3 in osteoblastic bone formation (Ghoul-Mazgar *et al.*, 2005; Hassan *et al.*, 2004, 2006). In addtion to osteoblastic bone formation, osteoclastic bone resorption is also an essential com- ponent of bone density regulation. However, we could not find the reports about the expression and/or role of Dlx3 in osteoclast. Therefore, in this study, we observed the expression pattern of Dlx3 during ostseoclast differentiation and examined the effect of overexpressed wild type *DLX3* (wtDlx3) and 4-BP DEL *DLX3* (TDO mtDlx3) on osteoclastogenesis.

Materials and Methods

Preparation of Dlx3 expression vectors

Human full length wild type DLX3 ORF expression clone (pReceiver-M12-wtDlx3) which contains N-terminal 3xFlag tag was purchased and sequence-verified (Genecopoeia; Germantown, Maryland, USA). TDO mtDlx3 expression vector was prepared by polymerase chain reaction (PCR)-amplified site-directed mutagenesis cloning into pReceiver-M12 vector. Following primers were synthesized at TaKaRa Korea (Seoul, Korea) and used for PCR-amplified site-directed mutagenesis; ① 5'-TGAATTCATGAGTGGCTCC-TTCGATC including EcoR1 restriction site (G/AATTC), ② 3'-TCTCGAGTCAGTACA CAGCCCCAGG including Xho1 restriction site (C/TCGAG), ③5'-ACAAGAAC *(gggg DEL)* AGGTGCCGCTGGAGCA, ④3'-GCTCCAGCGGCACCT *(cccc DEL)* GTTCTTGTAGAG. pReceiver-M12-wtDlx3 was digested by restriction enzyme EcoR1 and Xho1 (Roche; Mannheim, Germany). Enzyme-digested products were separated by gel running in 1.0% agarose gel and DNA fragments containing wtDlx3 ORF was eluted by using Gel Extraction Kit (Qiagen; Valencia, CA, USA). Two PCR reactions were performed from purified DNA fragments by using primers set of ① and ④ and of ② and ③. PCR-amplified products from each reactions were purified by gel extraction and put together in one tube. Then they were amplified by using primers ① and ②. PCR products were purified again by gel extraction and cloned into pReceiver-M12. Mutagenesis was verified by DNA sequencing (Macrogen; Seoul, Korea).

Cell culture

To observe the effect of wtDlx3 and TDO mtDlx3 on osteoclast differentiation, the monocyte cell line RAW264.7 was used. The cells were cultured in Dulbecco's modified Eagles medium (DMEM; Hyclone; Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, Gibco Invitrogen, Grand Island, NY, USA). Osteoclast differentiation was induced by culturing RAW264.7 cells in osteoclast differentiation medium consisted of minimum essential medium (MEM; Gibco Invitrogen) supplemented with 10% FBS, 200 ng/ml recombinant mouse RANKL (Cytolab; Rehovot, Israel) and antibiotics.

C2C12 cells were used to observe the effect of Dlx3 overexpression on the expression of RANKL, OPG and M-CSF. C2C12 cells were maintained in DMEM supple- mented with 10% FBS and antibiotics. To induce osteoblastic different iation, C2C12 cells were cultured in DMEM supplemented with 5% FBS, 100 ng/ml recombinant human bone morphogenetic protein 2 (BMP2; Wyeth Inc.; Cambridge, MA, USA) and antibiotics.

Transient transfection

Cells were plated at a density of 5×10^5 cells in 60 mm tissue culture dishes. After overnight incubation, cells were serum-starved. Plasmids used for transfection were pcDNA-3.1 for empty vector, pReceiver-M12-wtDlx3 and pReceiver-M12-TDO mtDlx3. DNA (8 μg/dish) and Lipofecta mine2000 (10 μl/dish; Invitrogen; Carlsbad, CA, USA) were combined and incubated for 20 minutes at room temperature. DNA-Lipofectamine complex was diluted in serum-free DMEM and added to serum-starved cells. After 4 hour incubation, culture medium was changed with DMEM supplemented with 5% FBS. After 24 hours, cells were used for experiments.

Stable transfection of RAW264.7 cells

For stable transfection, RAW264.7 cells were trypsinized and resuspended in Opti-MEM (Gibco BRL) supplemented with 10% FBS at the day of transfection. Cells (6×10^6 cells/100 mm culture dish) were subjected to reverse transfection with Lipofectamine2000. Plasmids used for transfection were pcDNA3.1, pReceiver-M12-wtDlx3 and pReceiver-M12-TDO mtDlx3. The day after transfection, cells were cultured in the complete growth medium containing 1 mg/ml G418 (Rhoche; Mannheim, Germany) to select those cells in which the foreign DNA was stably integrated into the genomic DNA. The cells were cultured to form colonies in the presence of G418 for three weeks with being fed every three days. The colonies obtained were resuspended as

single cells and mixed together. Then the cells were cultured at low-density in the presence of 400 μg/ml G418. For convenience, the RAW264.7 cells permanently expressing pcDNA3.1 were named RAW-ctrl; those expressing pReceiver-M12-wtDlx3, RAW-wt; and those expressing pReceiver-M12-TDO mtDlx3, RAW-mt.

Tartrate-resistant acid phosphatase (TRAP) staining

To induce formation of multinucleated osteoclasts, RAW-ctrl, RAW-wt and RAW-mt cells (2.5×10^4 cells/well) were seeded in a 24-well culture plate and cultured in the osteoclast differentiation medium for 4 to 6 days. Culture medium was changed every two days. At the end of culture period, the cells were washed twice with PBS, fixed and then stained with Leucocyte Acid Phosphatase kit (Sigma-Aldrich; St. Louis, MO, USA) according to manufacturer's instruction. The number of TRAP-positive cells with three or more nuclei was scored under the light microscope. The graphical presentation reflects the mean of three independent experiments and statistical significance was evaluated by unpaired Student's *t*-test.

Reverse transcription-polymerase chain reaction (RT-PCR)

Semiquantitative RT-PCR was performed for evaluating mRNA expression levels. Total RNA was isolated by using easy-blue RNA Extraction Reagent (iNtRON Biotechnology; Sungnam, Korea). Complementary DNA was synthesized from 2 μg of total RNA by using AccuPower RT Premix (Bioneer; Daejeon, Korea). PCR reactions were carried out in a final volume of 20 μl containing 1 U of i-star Taq DNA polymerase (iNtRON Biotechnology), 2 μl cDNA, 1× PCR buffer, 0.25 mM dNTP mixture and 10 μmol specific primers. PCR primers were synthesized by TaKaRa Korea. Mouse genes and their primer sequences used for PCR are as follows; M-CSF-forward (f) 5'-ACCATGGGGACAGACTCACTG-3', M-CSF-reverse (r) 5'-TCCCTCATGCTGCTCCACATG-3'; RANKL-f 5'-ATCAGAAGACAGCACTCACT-3', RANKL-r 5'-ATCTAGGACATCCATGCTAATGTTC-3'; OPG-f 5'-TGAGTGTGAGGAAGGG CGTTAC-3', OPG-r 5'-TTCCTCGTTCTCTCAATCTC-3'; RANK-f 5'-AAGATGGGTCCAGAAGACGGT-3', RANK-r 5'-CATAGAGTCAGTTCTGCTCG GA-3'; calcitonin receptor (CTR)-f 5'-TGAAAAGGCGGAATCT-3', CTR-r 5'-AGGAACATGTGCTTGTG-3'; vitronectin receptor integrin β_3 subunit (VNR)-f 5'-GCTCAGATGAGACTTTG-3', VNR-r 5'-ATCAACAATGAGCTGGA-3'; RhoA-f 5'-CGCTTTTGGGTACATGGAGT-3', RhoA-r 5'-TCTTTGAATTAGCGCCTGGT-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-f 5'-TCACCATCTTCCA GGAGCG-3', GAPDH-r 5'-CTGCTTACCACCTTCTTGA-3'. PCR products were separated by electrophoresis on 1.2% agarose gel and visualized under UV light after ethidium bromide staining.

Immunoblot analysis

After appropriate treatment, cells were washed with PBS and scraped into lysis buffer consisted of 10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM NaF, 0.2 mM Na_3VO_4, 1 mM phenylmethylsulphonylfluoride (PMSF), 1 μg/ml aprotinin, 1 μM leupeptin and 1 μM pepstatin, and sonicated briefly. Protein concentrations were determined using a modified Bradford method (Bio-Rad Laboratories; Hercules, CA, USA). Samples containing equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins separated in the gel were subsequently transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with the indicated primary antibody followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. Luminescence was detected using Supex reagent in a LAS1000 (Fuji PhotoFilm; Tokyo, Japan). Antibodies used for immunoblot analysis were as follows; mouse anti-Flag M2-HRP (Sigma-Aldrich), goat anti-Actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-Nuclear factor of activated T cells-c1 (NFATc1; Santa Cruz Biotechnology), goat anti-Cathepsin K (Santa Cruz), rat anti-CD9 (BD Biosciences Pharmingen; Chicago, IL, USA), goat anti-mouse HRP-conjugated IgG (Zymed; S. San Francisco, CA, USA) and rabbit anti-goat HRP-conjugated IgG (Zymed).

To observe endogenous Dlx3 protein expression in RAW-264.7 cells, nuclear extracts were prepared. Cells were seeded at a density of 2×10^6 cells/dish in 100 mm culture dishes and cultured in osteoclast differentiation medium for the times indicated. Then, nuclear extracts were prepared by using NE-PER Nuclear and Cytoplasmic Extraction Reagents (PIERCE Biotechnology; Rockford, IL, USA) according to the manufacturer's instruction. Briefly, cell pellet was obtained by centrifugation at 500× g for 3 minutes, resuspended in Cytoplasmic Extraction Reagent I and incubated on ice for 10 minutes. Then Cytoplasmic Extraction Reagent II was added and incubated on ice for 1 minute. After centrifugation at 16,000× g for 5 minutes, the supernatant (cytoplasmic fraction) was collected and pellet was resuspended in Nuclear Extraction Reagent and incubated on ice for 40 minutes with intermittent vortexing. After centrifugation at 16,000× g for 10 minutes, the supernatant (nuclear fraction) was collected and protein concentrations was determined. Then immunoblot analysis was performed with cytosolic fractions and nuclear fractions as described above.

Results & Discussion

Both wtDlx3 and TDO mtDlx3 overexpression enhanced RANKL-induced osteoclastogenesis in RAW264.7 cells

To examine whether Dlx3 was expressed during RANKL-induced osteoclast differentiation, we used RAW264.7 cells. RAW264.7 is a murine macrophage cell line and widely

used for studying osteoclast differentiation due to its potential of osteoclast-like multinucleated cell formation (Collin-Osdoby *et al.*, 2003). As expected, Dlx3 was mainly detected in nuclear fraction and Dlx3 expression increased slightly after RANKL treatment for 1 day and peaked when the fusion of prefusion osteoclasts actively progressed (Fig. 1).

In order to examine the effect of overexpressed Dlx3 proteins, we established stable cell lines that overexpress wtDlx3 or TDO mtDlx3 using RAW264.7 cells. Expression levels of Dlx3 mRNA and protein in stable transfectants were confirmed by RT-PCR and immunoblot analysis (Fig. 2A). wtDlx3 expressed in RAW-wt appeared in several bands. In addition, the expression level of exogenous Dlx3 protein was much lower in RAW-wt than in RAW-mt. When these cells were cultured in the presence of RANKL, the number and size of TRAP-positive multinucleated cells increased in both RAW-wt and RAW-mt compared to RAW-ctrl cells (Fig. 2B & 2C). Although both of wtDlx3 and mtDlx3 accelerated osteoclastogenesis, RAW-mt formed more multinucleated cells than wtDlx3. Since protein level of TDO mtDlx3 was much higher than that of wtDlx3, it is not likely that TDO mtDlx3 has higher potential than wtDlx3 to enhance RANKL-induced osteoclastogenesis in RAW264.7 cells.

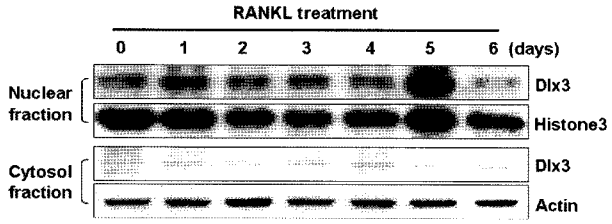

Fig. 1. Dlx3 is expressed during osteoclast differentiation. RAW-264.7 cells were cultured in the presence of 200 ng/ml RANKL for the times indicated. Cell lysates were prepared and separated to cytosolic and nuclear fractions, and then immunoblot analysis was performed.

To examine the expression pattern of osteoclast differentiation-associated genes, we performed RT-PCR and immunoblot analysis. The mRNA expression levels of RANK, VNR and CTR were higher in RAW-wt and RAW-mt compared to RAW-ctrl, coinciding with TRAP staining results (Fig. 3A). RhoA was used as a negative control whose expression did not change during osteoclast differentiation. Cathepsin K protein level was also higher in RAW-wt and RAW-mt compared to RAW-ctrl (Fig. 3B). Increased expression of CD9, a molecule involved in osteoclast fusion regulation (Ishii *et al.*, 2006), was also observed in RAW-wt and RAW-mt. The expression of osteoclast differentiation-associated genes is known to be regulated by several key transcription factors. NFATc1 is one of the most strongly induced

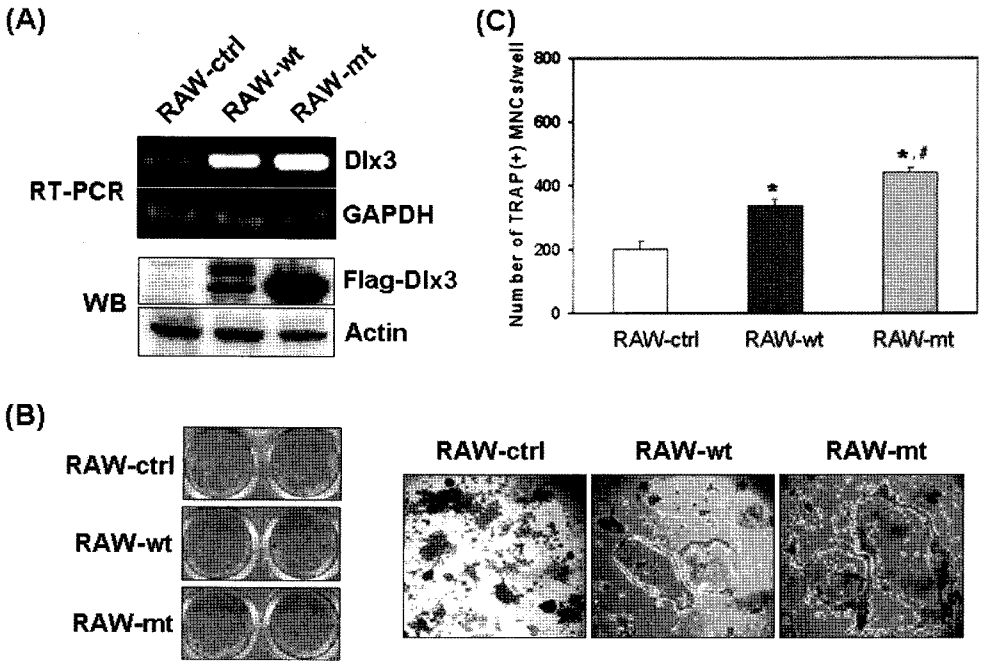

Fig. 2. Overexpression of wtDlx3 and TDO mtDlx3 promote osteoclastogenesis in RAW264.7 cells. RAW264.7-stable transfectants expressing pcDNA3.1 (RAW-ctrl), wtDlx3 (RAW-wt) and TDO mtDlx3 (RAW-mt) were generated. (A) Overexpression of Dlx3 was confirmed by RT-PCR (*upper panel*) and immunoblot analysis (*lower panel*). (B, C) Stable transfectants were cultured for 4 days in the presence of 200 ng/ml RANKL and TRAP staining was performed. (B) Images of cell culture plate (*left panel*) and of individual wells (*right panel;* × 100) shows that overexpression of wtDlx3 or TDO mtDlx3 enhanced the formation of multinucleated TRAP-positive cells. (C) The number of TRAP-positive multinucleated cells with three or more nuclei was counted. * significant difference from RAW-ctrl ($p < 0.01$), # significant difference from RAW-wt ($p < 0.01$). MNCs: multinucleated cells

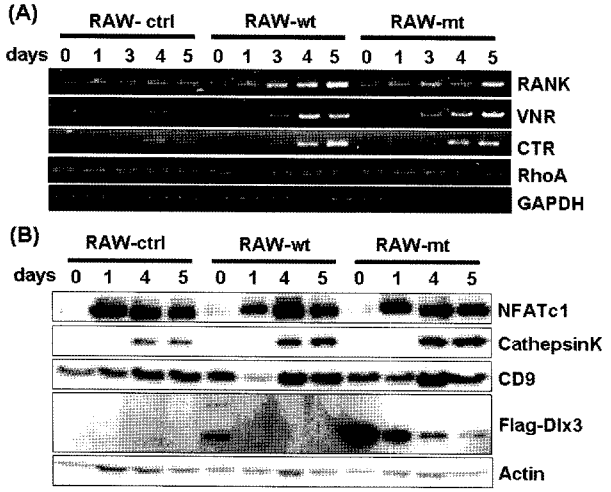

Fig. 3. Overexpression of wtDlx3 or TDO mtDlx3 enhanced the expression of osteoclast differentiation-associated genes. RAW stable transfectants were cultured in the presence of 200 ng/ml RANKL for the times indicated. Then RT-PCR (A) or immunoblot analysis (B) was performed. (A) RAW-wt and RAW-mt cells showed higher mRNA levels of osteoclast differentiation marker genes such as RANK, vitronectin receptor β_3 subunit (VNR) and calcitonin receptor (CTR) than RAW-ctrl. RhoA is a negative control. (B) Although NFATc1 protein level was not increased, the protein levels of cathepsin K and CD9 were increased by overexpression of wtDlx3 and TDO mtDlx3.

transcription factors following RANKL stimulation and a critical regulator of osteoclastogenesis (Northrop *et al.*, 1994; Matsuo *et al.*, 2004). Since ectopic expression of NFATc1 caused the osteoclast precursor cells to efficiently differentiate into osteoclasts in the absence of RANKL, it is suggested that NFATc1 might play an integral role in RANKL-induced transcriptional program during osteoclast differentiation. MITF is a basic helix-loop-helix leucin zipper protein and a critical role of MITF in osteoclast differentiation is provided by the severe osteopetrotic phenotype in mice homozygous for *mitf* alleles (Marks and Walker, 1981). Interactions between MITF and PU.1 are known to be necessary for the ability of MITF to selectively regulate target genes during osteoclastogenesis (Luchin *et al.*, 2001). In addition, a recent report demonstrated that MIFT and PU.1 recruit NFATc1 to promoters of osteoclast differentiation-associated genes during osteoclast differentiation (Sharma *et al.*, 2007). Therefore, we examined the expression levels of NFATc1, MITF and PU.1. The protein level of NFATc1 in RAW-wt and RAW-mt was not higher than that in RAW-ctrl (Fig. 3B). In addition, mRNA levels of NFATc1, MITF and PU.1 was not affected by Dlx3 overexpression (data not shown). These results indicate that enhancement of osteoclast formation by Dlx3 does not depend on the enhanced expression of these transcription factors.

Even though exogenous Dlx3 expression is driven by CMV promoter, the level of exogenously expressed Dlx3 proteins decreased in accordance with the progression of osteoclastogenesis (Fig. 3B). It is not clear now why exogenous Dlx3 level was down-regulated during osteoclastogenesis.

Overexpression of wtDlx3 increased RANKL expression in C2C12 cells while that of TDO mtDlx3 did not

Induction of osteoclast differentiation from hematopoietic progenitor cells needs the supply of RANKL and M-CSF from the microenvironment such as bone marrow stromal cells and osteoblasts. In addition, osteoclast differentiation is critically regulated by the balance between RANKL and OPG (Nagai and Sato, 1999). To define whether there were any differences in the expression of these genes between wtDlx3-overexpressing and TDO mtDlx3-overexpressing cells, we transiently transfected C2C12 cells with wtDlx3 and mtDlx3 expression vectors and cultured them in the presence of 100 ng/ml BMP2 for 3 days. Since previous reports have shown that BMP2 enhanced 1,25-dihydroxyvitamine D_3-induced osteoclast formation in coculture of C2C12 cells and spleen cells (Otsuka *et al.*, 2003) and BMP2 alone increased RANKL expression in osteoblast-like cells (Koide *et al.*, 1999), we observed RANKL expression in C2C12 cells overexpressing wtDlx3 or TDO mtDlx3 in the presence of BMP2. RT-PCR results showed that mRNA expression of RANKL increased while that of OPG, a decoy receptor for RANKL, decreased slightly in wtDlx3-overexpressed cells, shifting the ratio of RANKL to OPG to favorable condition for osteoclast formation (Fig. 4). In contrast, overexpression of TDO mtDlx3 did not affect significantly the expression of RANKL and OPG. Since regulation of RANKL or OPG expression by wtDlx3 overexpression was not obvious in earlier time point than 3 days (data not shown), it is unlikely that wtDlx3 directly

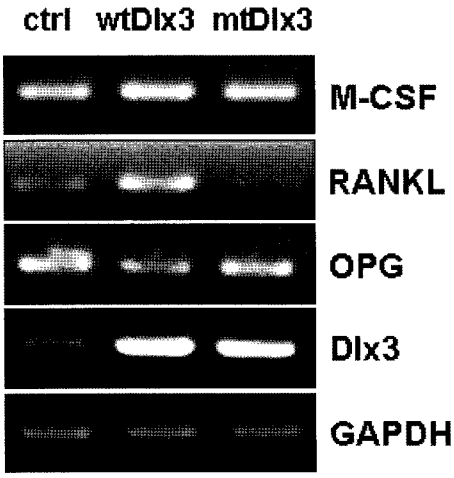

Fig. 4. Overexpression of wtDlx3, but not that of TDO mtDlx3, induced mRNA expression of RANKL. C2C12 cells were transiently transfected with wtDlx3 and TDO mtDlx3 expression plasmids. After 24 hours, cells were incubated in the presence of 100 ng/ml BMP2 for 3 days. Then total RNA was isolated and semiquantitative RT-PCR was performed.

regulates the transcription of RANKL or OPG in these cells. The expression of M-CSF did not change significantly in wtDlx3- or mtDlx3-overexpressing cells. These results suggest that inability of TDO mtDlx3 to regulate expression of RANKL and OPG may contribute to increased bone density phenotype in TDO syndrome patients.

Taken together, we demonstrated in this study that Dlx3 plays a role as a positive regulator of osteoclast differentiation via up-regulation of osteoclast differentiation-associated genes expression in osteoclasts, as well as via increase in the ratio of RANKL to OPG in osteoblastic cells.

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