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**Expression profile of RhoGTPases and RhoGEFs during RANKL-stimulated osteoclastogenesis:  
identification of essential genes in osteoclasts.**

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**CONFLICT OF INTEREST**

All authors have no conflict of interest.

## **MICRO ABSTRACT.**

RhoGTPases regulate actin cytoskeleton dynamics, a key element in osteoclast biology. We identified three novel genes induced during RANKL-stimulated osteoclastogenesis among RhoGTPases and their exchange factors that are essential in osteoclast biology.

## **ABSTRACT**

**Introduction.** During the process of differentiation, adhesion to the bone matrix or osteolysis, the actin cytoskeleton of osteoclasts undergoes profound reorganization. RhoGTPases are key regulators of actin dynamics. They control cell adhesion, migration and morphology through their action on actin cytoskeleton. In mouse, there are 18 low molecular weight RhoGTPases. They are activated by guanine nucleotide exchange factors: the RhoGEFs. There are 76 RhoGEFs in mouse: 65 belong to the Dbl family and 11 to the CZH family. In order to identify novel genes among RhoGTPases and RhoGEFs important in osteoclasts, we establish the expression profiles of the complete families of RhoGTPases and RhoGEFs during RANKL-stimulated osteoclastogenesis.

**Materials and Methods.** RAW264.7 cell line, mouse bone marrow macrophages and hematopoietic stem cells were used as precursors for RANKL-induced osteoclastogenesis. Gene arrays and real-time quantitative PCR analyses were performed to establish the transcription profiles of RhoGTPase and RhoGEF genes during differentiation. Small hairpin RNAs were used to knock down genes of interest.

**Results.** Of the 18 RhoGTPases and 76 RhoGEFs, the expression of three genes was up-regulated by RANKL: the RhoGTPase RhoU/Wrch1, the Dbl family exchange factor Arhgef8/Net1 and the CZH family exchange factor Dock5. The inductions were observed in gene array and real-time quantitative PCR experiments performed in RAW264.7 cells. They were further confirmed in bone marrow macrophages and hematopoietic stem cells. Silencing of Wrch1 and Arhgef8 expression severely inhibited differentiation and affected osteoclast morphology. Dock5 suppression was lethal in osteoclast precursors while having no effect in fibroblasts.

**Conclusion.** We have identified three genes among RhoGTPase signaling pathways that are up-regulated during RANKL-induced osteoclastogenesis. These genes are novel essential actors in osteoclasts, most likely through the control of actin cytoskeleton dynamics.

## **KEY WORDS**

Bone; Osteoclast; Actin; Gene Array; Transcription.

## INTRODUCTION

Osteoclasts are multinucleated cells specialized in bone resorption. Upon the differentiation process stimulated by the cytokine RANKL, osteoclasts activate a complex transcriptional program (1) while they undergo profound actin cytoskeleton reorganization (2, 3). Osteoclasts anchor to the bone matrix through the sealing zone, a dynamic actin ring that delineates the resorption lacuna (4). The importance of actin-based structures in osteoclast function suggests an essential role for RhoGTPases in these cells. Through the control of actin dynamics, small GTPases of the Rho family regulate many cellular processes such as cell adhesion, migration and morphology (5) as well as vesicular trafficking (6, 7). RhoGTPases cycle between an inactive GDP- and an active GTP-bound state. The Rho family of small GTPases comprises 18 members involved in actin cytoskeleton reorganization (8), namely RhoA, B and C, Rac1, 2 and 3, RhoG, Cdc42, RhoQ/TC10, RhoJ/TCL, RhoV/Chp1, RhoU/Wrch1, Rnd1, RhoN/Rnd2, RhoE/Rnd3, RhoD, RhoH and RhoF/Rif. In the cell, RhoGTPases are activated by Guanine nucleotide Exchange Factors (RhoGEFs) that catalyze the exchange of GDP for GTP (9). There are more than 70 RhoGEFs in mammals, that belong to two families: over 60 Dbl-related proteins (9) and 11 DOCK-related CZH proteins (10). Different studies highlighted the importance of RhoGTPases in cell differentiation, including the development of the nervous system (11) and B-cell maturation (12). RhoGTPases are also essential for skeletal muscle and trophoblast differentiation (13, 14). Interestingly, myoblasts, trophoblasts and osteoclasts are the only cell types that undergo cell fusion during normal differentiation. Since the cell fusion process implies important actin cytoskeleton remodeling and involves RhoGTPases in myoblasts and trophoblasts differentiation (15-17), it suggests that Rho signaling pathways could also control the fusion of osteoclast precursors.

Only few RhoGTPases and RhoGEFs have been studied in osteoclasts. Several studies reported the role of RhoA, Rac1, Rac2 and Cdc42 in the control of osteoclast resorbing activity or adhesion to the bone matrix (4, 18-23). Moreover, farnesyl pyrophosphate synthase is the specific target of nitrogen-containing bisphosphonates, the most widely used treatment for osteoporosis. Interestingly, this enzyme is essential for the prenylation and thereby the activity of RhoGTPases such as RhoA, Rac1 and Cdc42 (24). Bisphosphonate treatment results in the loss of osteoclast activity and induction of apoptosis, possibly through the inactivation of RhoGTPases (25). The RhoGEF Vav3 is also required for the osteoclastic bone matrix degradation (26).

Despite the clear implication of RhoGTPase signaling pathways in osteoclast biology, no information is available on the role of most RhoGTPases and RhoGEFs during osteoclast differentiation and bone resorption. In this study, we established the expression profile of RhoGTPases and RhoGEFs using Affymetrix DNA micro

arrays and real-time quantitative PCR during osteoclast differentiation. We performed differentiation *in vitro* using RAW264.7 cells and *ex vivo* using bone marrow macrophages and hematopoietic stem cells as osteoclast precursors. We identified three genes up-regulated by RANKL and further demonstrate they are essential in osteoclasts using small hairpin RNAs (shRNAs) to inhibit their expression.

## **MATERIALS AND METHODS**

### **Cells and microscopy analyses.**

Cells were cultured in a humidified incubator (5% CO<sub>2</sub> in air) at 37°C. NIH3T3 mouse fibroblastic and 293T human embryonic kidney cell lines were cultured in the following growth medium: DMEM containing 10% fetal calf serum (Hyclone) with 2 mM glutamine. Mouse monocytic RAW264.7 cells (a gift from P. Jurdic, Lyon, France) and bone marrow-derived cells were grown in the following growth medium: alpha-MEM containing 10% heat-inactivated fetal calf serum (Hyclone) with 2 mM glutamine. For osteoclast differentiation, RAW264.7 cells were seeded at 5x10<sup>4</sup> cells per well in a 6-well plate with 25 ng/ml of RANKL (Peprotech). Medium was changed every second day.

Bone marrow was collected from 4-5 week old C57BL/6 mice. Lin-CD117<sup>+</sup> hematopoietic stem cells (HSCs) (27) were isolated from total bone marrow by depletion of differentiated cells (CD5<sup>+</sup>, CD45R<sup>+</sup>, CD11b<sup>+</sup>, Ly-6G<sup>+</sup>, 7-4<sup>+</sup> and Ter-119<sup>+</sup>) with antibody-coupled magnetic beads using the cell lineage depletion kit provided by Miltenyi Biotec (Bergisch Gladbach, Germany) on a MACS separator system, followed by positive selection of CD117<sup>+</sup> cells with Miltenyi Biotec anti-CD117 coupled magnetic beads. To purify Bone Marrow Macrophages (BMMs), non-adherent cells from total bone marrow (2.5x10<sup>6</sup> per well in a 6-well plate) were cultured with 50 ng/ml M-CSF (Peprotech) for 48 hours. Adherent cells were used as BMMs after washing non-adherent cells. Lin-CD117<sup>+</sup> hematopoietic stem cells (HSCs) and adherent Bone Marrow Macrophages (BMMs) were used as osteoclast precursors. For osteoclast differentiation, HSCs and BMMs were further cultured in the presence of 50 ng/ml M-CSF and 100 ng/ml RANKL. Medium was changed every second day.

After fixation in 10% formalin for 10 minutes and washing with ethanol-acetone (1:1 volume-volume), osteoclast differentiation was assessed by staining for the tartrate-resistant acid phosphatase (TRAP), a marker enzyme for osteoclasts, with 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml fast red violet LB salt in 0.1 M sodium acetate buffer pH 5 containing 50 mM sodium tartrate. Nuclei were stained with Hoechst dye and actin with rhodamine-labeled phalloidin (Sigma Aldrich). Cells were observed using a DMR B microscope (Leica Microsystems). Images were captured with a MicroMax 1300 HS (B/W) cooled charge coupled device camera or CoolSnap Color Camera (Roper Scientific) using a MetaMorph control program (Princeton Scientific Instruments). TRAP-positive cells with more than 3 nuclei were considered as osteoclasts. The frequency of osteoclast formation was calculated as the fusion index according to the formula: Fusion Index (%)=(number of nuclei in multinucleated cells)x100/(total number of nuclei). In each of three independent experiments and for each shRNA, the fusion indexes were determined in 12 to 15 microscopic fields of 0.6 mm<sup>2</sup>, counting a total of

at least 2500 nuclei. Statistical analyses were performed by analysis of variance (ANOVA), using Fisher's Least Significant Difference (LSD) procedure to discriminate among means. Differences were considered significant for  $P < 0.05$ . For the same shRNA, the mean fusion indexes did not vary significantly between experiments ( $P > 5\%$ ) whereas the shRNA type correlated significantly with the fusion index ( $P = 0.0002$ ).

### **Real-time PCR analyses**

DNaseI-treated total RNAs were extracted using the High Pure RNA Isolation kit (Roche Diagnostics). To generate cDNA, RNA was primed with 10-mer random primers and reverse transcription catalyzed using Superscript II reverse transcriptase (Invitrogen). Quantitative PCR was performed with a Light Cycler (Roche Diagnostics) or an Mx3000p PCR system (Stratagene) using the Platinum Taq DNA polymerase (Invitrogen) and SYBR Green I (Bio Wittaker) as described (28). All primer pairs used in this study are listed in Table 1. For each primer set, specificity was assessed by purification and sequencing of the PCR product. All real time PCR measures to quantify cDNAs were done in triplicate and the 95% confidence limits of the ratios to Gapdh were determined by Student's *t*-test. In each experiment, cDNA levels were considered changing between the two conditions tested (with and without RANKL) when the 95% confidence limits of their ratios to Gapdh were not overlapping. All genes tested consistently gave the same results (changing or not changing,) in the three experiments. Data shown are representative of one experiment. The values of the cDNA ratios to Gapdh are not intended to be compared from one gene to another.

### **Gene Array Studies.**

Total RNA from RANKL-treated and control RAW264.7 cells was prepared using the guanidium thiocyanate technique as described previously (29). Total RNA quality was ascertained using the Agilent Bioanalyser 2100 using the NanoChip protocol. Total RNA was labeled using the Affymetrix One Cycle cRNA synthesis kit (Millenium Sciences) and cleaned using the Affymetrix GeneChip Sample Cleanup kit (Millenium Sciences). Resultant cRNA was labeled with biotin using the Affymetrix IVT labeling kit (Millenium Sciences) and cleaned using the above Cleanup kit. A total of 20  $\mu\text{g}$  of labeled cRNA was then fragmented to the 50-200 base pair size range and quality control checked with the Agilent Bioanalyser 2100 using the NanoChip protocol. For hybridization of the Mouse 430 version 2.0 GeneChip, cRNA was included at 0.05  $\mu\text{g}/\mu\text{l}$  in 1x Hybridization Buffer (100 mM MES, 1M NaCl, 20 mM EDTA, 0.01% Tween-20), 0.1 mg/ml Herring Sperm DNA, 0.5 mg/ml BSA, and 7% DMSO. Hybridization was performed at 45°C for 16 hours in an oven with a rotating wheel. After hybridization, chips were washed using the appropriate fluidics script in the Affymetrix Fluidics Station 450 and

scanned using the Affymetrix GeneChip Scanner 3000. Scanned output files were analyzed using Micro Array Suite software (Affymetrix).

#### **Western blot analyses.**

Polyclonal antiserum against Arhgef8/Net1 was from Santa Cruz Biotechnology. Polyclonal anti-Gapdh antibody was described earlier (7). Horseradish peroxidase-conjugated secondary antibodies were from Amersham Bioscience and detected using enhanced chemiluminescence from Perkin Elmer Life Science. RAW264.7 whole cell extracts were prepared in Laemmli sample buffer, resolved on SDS-PAGE and electrotransferred on nitrocellulose membranes. Immunoblotting was then performed according to manufacturer instructions.

#### **Small hairpin RNA retroviral vector construction and RAW264.7 cell infection.**

19 mer small hairpin RNA (shRNA) target sequences (Wr-1, Wr-2, Gef8-1, Gef8-2, Dk5-1 and Dk5-2, Fig. 3A) were selected in mouse Wrch1, Arhgef8 and Dock5 open reading frames and the 65-mer sense and antisense strands of DNA oligonucleotides were designed according to the Clontech Bioinformatics Data Server. Oligonucleotides were synthesized by Invitrogen, annealed and cloned into pSIREN-RetroQ vector containing a puromycin resistance selection marker according to manufacturer's instructions (Clontech). The pSIREN-RetroQ-Luc vector (Clontech) targeting firefly Luciferase was used as control. We performed retrovirus packaging by cotransfection of the pSIREN-RetroQ vectors, the Friend MLV-based Gag-Pol expression vector pC57GP (30) and the VSV-G envelope glycoprotein expression vector pCSIG (31) (gifts from Marc Sitbon, Montpellier, France) into 293T cells using JetPEI (QBiogen). Viral supernatants were harvested 3 days post transfection and filtered through a 0.45  $\mu\text{m}$  pore size filter. For infections, RAW264.7 and NIH3T3 cells were plated at  $2 \cdot 10^5$  cells per 6-cm dish. The next day the medium was replaced for 4 hours with 1,5 ml of viral supernatant and 0,5 ml of growth medium, containing 8  $\mu\text{g/ml}$  polybrene. Cells were left to recover in growth medium for 24 hours and infected cells were then selected by addition of puromycin (3 $\mu\text{g/ml}$ ) for another 24 hours. Infected RAW264.7 and NIH3T3 cells were scrapped and reseeded in growth medium at  $5 \cdot 10^4$  cells per well of a 6-well plate for further analyses.

## **RESULTS**

### **RhoGTPase expression profiles during osteoclast differentiation.**

To identify RhoGTPases genes up-regulated during RANKL-induced osteoclastogenesis, we designed 18 primer sets to amplify each RhoGTPase (Table 1). For all primer sets chosen for this study, the PCR efficiency was above 95% (not shown). As Rho family members can have up to 70% identity at the nucleic acid level, primers were chosen in the most divergent regions and specificity was verified by sequencing the PCR products. These primers were then used to quantify RhoGTPase mRNAs during osteoclast differentiation of RANKL-treated RAW264.7 cells. For each sample, Gapdh was used as an internal house keeping gene control.

When RAW264.7 cells were treated with RANKL, fusion occurred between day 3 and 4 and flat round TRAP-positive multinucleated osteoclasts appeared between day 4 and 5. Total mRNA was prepared at days 0, 3, 6 and 8 after RANKL addition and mRNA of each RhoGTPase was quantified relative to the amount of Gapdh mRNA in the same sample. The increasing levels of Src and TRAP mRNAs were used as markers for osteoclast differentiation (Fig. 1A). Interestingly, we observed an important accumulation of RhoU/Wrch1 mRNA in RANKL-treated cells (Fig. 1B). The level of Wrch1 mRNA increased about 100 fold in RAW264.7 cells after 3 days of RANKL treatment and remained high up to day 8, while the level of Cdc42, a RhoGTPase related to Wrch1 (32), remained constant throughout osteoclast differentiation (Figs. 1B and 1C). For 12 RhoGTPases, namely Rac2, RhoA, RhoB, RhoC, RhoG, Cdc42, RhoF/Rif, Rac1, RhoQ/TC10, Rnd1, RhoN/Rnd2 and RhoV/Chp1, we did not observe any significant variation of mRNA levels during osteoclast differentiation (Fig. 1B). Finally, the mRNAs of 5 GTPases were not detected: RhoD, RhoH, Rac3, RhoJ/TCL and RhoE/Rnd3 (not shown).

These results show that Wrch1 gene expression is strongly up-regulated during osteoclastogenesis stimulated by RANKL in RAW264.7 cells; 5 RhoGTPases are not expressed and mRNA levels of 12 RhoGTPases are not affected by RANKL treatment in RAW264.7 cells.

### **RhoGEF expression profiles during osteoclast differentiation.**

In cells, RhoGTPases are activated by guanine nucleotide exchange factors. We thus explored the expression of RhoGEFs during osteoclast differentiation. As the mouse genome contains 65 Dbp related and 11 DOCK related RhoGEF genes, we performed DNA micro array analyses to examine their expression during osteoclast differentiation. We identified probe sets for the 76 mouse RhoGEFs in the mouse genome A430-2 Affymetrix oligonucleotide chip (Table 2). BLAST searches were performed on each probe set through Affymetrix NetAffx Analysis Center to verify its identity to the Unigene RhoGEF transcripts. To analyze

RhoGEF expression during osteoclast differentiation, RNAs were isolated from RAW264.7 cells with or without treatment of RANKL for 72 hours. Labeled cRNAs were prepared to hybridize Affymetrix Mouse genome A430-2 oligonucleotide chips.

In agreement with earlier reports, we detected the up-regulation of known RANKL-induced genes, such as TRAP, Cathepsin K, Src and MMP-9 (Table 3). Moreover and consistent with the above RT-PCR studies, we observed a strong up-regulation of *Wrch1* in RANKL-treated RAW264.7 cells (Table 3), while the RhoGTPases TCL, RhoD, RhoH, Rnd3 and Rac3 were absent (not shown). These results warranted our analysis. Since  $\beta$ -actin and *Gapdh* control house keeping genes showed a 1.4 to 1.6 fold increase respectively in cells treated with RANKL, we chose to define as RANKL-induced RhoGEFs those with over 3 fold up-regulation in RANKL-treated as compared to control cells. Of the 76 RhoGEFs, 42 RhoGEFs were expressed and 34 genes were not (Table 2), based on the absolute calls determined by Micro Array Suite software provided by Affymetrix for data analysis. Of the 42 RhoGEF genes expressed, 7 genes were up-regulated in RANKL-treated cells: *Dock5*, *Fgd6*, *Geft*, *Farp2*, *Vav3*, *Arhegf8/Net1* and *Arhegf12/Larg* (Table 4).

To confirm the DNA micro array analyses, we designed primer sets to amplify and quantify the 7 RhoGEFs identified above (Table 1). *Itsn2* was used as a control as its expression is not modified in RANKL-treated cells (Table 4). The specificity of the primers was verified by sequencing the PCR products and PCR efficiency was above 95% for all primer sets (not shown). Real-time PCR analyses confirmed that the 7 RhoGEFs identified by DNA micro arrays were up-regulated during osteoclastogenesis induced by RANKL in RAW264.7 cells (Fig. 1D). Also expected from the DNA chip experiment, the level of *Itsn2* did not vary significantly between RANKL-treated and -untreated cells (Table 4 and Fig. 1D). We also verified the induction of *Src*, TRAP (not shown) and *Wrch1* (Fig. 1D). Western blot analyses in RAW264.7 showed that *Arhegf8* protein expression is induced by RANKL (Fig. 1E).

By combining gene array and real-time PCR analyses, we show here that 7 out of the 76 mouse RhoGEFs genes are up-regulated during osteoclast differentiation induced by RANKL in RAW264.7 cells.

#### **RANKL-dependent induction of *Wrch1* and RhoGEFs in osteoclasts differentiated *ex vivo*.**

To extend the results obtained in RAW264.7 cell line, we differentiated osteoclasts *ex vivo* using two types of precursor cells isolated from mouse bone marrow: lin-CD117+ hematopoietic stem cells (HSCs) or bone marrow macrophages (BMMs). Multinucleated TRAP positive cells appeared at day 5 to 6 after RANKL addition in BMM cultures and at day 6 to 7 after RANKL addition in HSC cultures (shown for HSCs in Fig. 2A

and B). Osteoclast differentiation was monitored by the increase in Src and TRAP mRNAs levels, as determined by real-time PCR (shown for HSCs in Fig. 2C).

We observed the up-regulation of *Wrch1* gene expression during HSC and BMM osteoclastogenesis while the level of *Cdc42* did not change significantly (Fig. 2D for HSCs and 2E for BMMs). *Arhgef8* and *Dock5* were also up-regulated during osteoclastogenesis in both *ex vivo* differentiation systems (Fig. 2F and G). By contrast, *Arhgef12*, *Farp2* and *Fgd6* genes that were significantly up-regulated in RANKL-treated HSCs (Fig. 2F) did not vary significantly in BMMs (Fig. 2G). The expression of *Vav3* was not significantly modified by RANKL treatment in HSCs and BMMs (Fig. 2F and G). Finally, *Gef2* was not detected in HSCs and BMMs treated with M-CSF alone or M-CSF and RANKL (not shown) whereas it was expressed in RAW264.7 cells (Fig. 1D and Table 4).

These results obtained with HSCs and BMMs, two types of bone marrow-derived osteoclast precursors, confirmed that the RhoGTPase *Wrch1* and the RhoGEFs *Arhgef8* and *Dock5* are up-regulated during RANKL-induced osteoclastogenesis.

#### ***Wrch1*, *Arhgef8* and *Dock5* silencing affects osteoclast differentiation.**

To test the importance of *Wrch1*, *Arhgef8* and *Dock5* in osteoclasts, we performed small hairpin RNA-induced gene silencing. We selected 2 target sequences in the open reading frames of each gene (Fig. 3A) and generated small hairpin RNA expression vectors. Firefly Luciferase specific shRNA was used as control (Fig. 3A). After infection of RAW264.7 cells and selection of infected cells, cells were reseeded and grown in the presence of RANKL to induce osteoclast differentiation. We monitored the efficiency of gene silencing by quantitative RT-PCR throughout osteoclast differentiation. *Wr-1* and *Wr-2* shRNAs efficiently silenced *Wrch1* gene expression throughout osteoclast differentiation (Fig. 3B) and so did *Gef8-1* and *Gef8-2* toward *Arhgef8* (Fig. 3C). To evaluate the effect of *Wrch1* and *Arhgef8* gene silencing on osteoclast differentiation, RAW264.7 expressing the different shRNAs were fixed after 5 days of exposition to RANKL, stained for TRAP and DNA and the fusion indexes were determined as described in Materials and Methods. *Wrch1* and *Arhgef8* silencing led to a strong diminution of the fusion indexes (Fig. 3D). The average fusion index was  $13.6 \pm 0.6$  % in Luciferase shRNA-expressing cells, it dropped down to  $2.9 \pm 0.8$  % and  $4 \pm 0.9$  % in *Wr-1* and *Wr-2* shRNA-expressing cells respectively, and down to  $6.0 \pm 1.2$  % and  $6.5 \pm 1.4$  % in *Gef8-1* and *Gef8-2* shRNA-expressing cells respectively. Osteoclasts expressing Luciferase shRNA did not show any morphological changes as compared to control uninfected cells (Fig. 3E and F). By contrast, osteoclasts differentiated from *Wrch1* or *Arhgef8* shRNA-expressing cells were abnormally spread and had long peripheral extensions (Shown

for Wr-1 and Gef8-2 in Fig. 3G and H). Contrarily to Wrch1 and Arhgef8, Dock5 silencing was highly toxic in RAW264.7. Cells expressing Dk5-1 and Dk5-2 died massively within 3 days after infection (not shown), therefore further studies could not be performed in these cells. To assay for cell type specificity of Dock5 silencing cytotoxic effect, Dk5-1 and Dk5-2 were expressed in NIH3T3 fibroblastic cells. Dock5 was efficiently silenced by Dk5-1 and Dk5-2 as shown by quantitative RT-PCR (Fig. 3I). Interestingly and in contrast with RAW264.7 cells, Dock5 silencing was not cytotoxic in NIH3T3 and cells did not show any noticeable changes in actin structure as compared to control Luciferase shRNA expressing cells (shown for Dk5-2 in Fig. 3J and K). These results show that Dock5 is essential in RAW264.7 cells and that it is not an essential house-keeping gene as its silencing has no effect in NIH3T3 fibroblastic cells.

These results show that the three genes that we identified and that are up-regulated during RANKL-stimulated osteoclastogenesis are essential in osteoclasts: Wrch1 and Arhgef8 silencing inhibited cell fusion and Dock5 silencing was cytotoxic in RAW264.7 monocytic cells, while having no effect in fibroblasts.

## DISCUSSION

In this report, we studied the expression of 18 low molecular weight RhoGTPase and 76 RhoGEF genes during RANKL-stimulated osteoclastogenesis. We demonstrated that the expression of three genes is up-regulated during RANKL-dependent osteoclast differentiation: one RhoGTPase, RhoU/Wrch1, one Dbl related RhoGEF, Arhgef8/Net1 and one CZH family RhoGEF, Dock5. Gene silencing by small hairpin RNA expression confirmed these genes are essential in osteoclasts.

RANKL treatment triggers a complex transcriptional program in RAW264.7 cells that leads to osteoclast differentiation *in vitro*. Large scale transcriptional analyses have proven suitable for the identification of genes potentially important in osteoclasts (1). In RAW264.7 cells, we observed consistent results between gene array analysis and quantitative RT-PCR experiments of RANKL up-regulated genes among RhoGTPases and RhoGEFs, using TRAP and Src as osteoclast differentiation markers. Nevertheless, RAW264.7 is an immortalized cell line derived from macrophages transformed with the Abelson mouse leukemia virus. Consequently, to minimize artifacts that may arise from using a cell line, we further confirmed our results in two *ex vivo* osteoclast differentiation systems, based on mouse bone marrow-derived osteoclast precursors. This allowed us to confirm that RhoU/Wrch1, Arhgef8/Net1 and Dock5 genes are up-regulated during osteoclastogenesis. We could only verify the induction of Arhgef8 at the protein level because of the lack of antibodies against Wrch1 and Dock5. Although mRNA levels do not necessarily reflect protein levels, the suppression of gene expression by shRNAs was a powerful tool to overcome this problem and confirm the importance of these genes in osteoclasts.

During this study, we found a very strong up-regulation of RhoU/Wrch1 gene expression. Wrch1 suppression severely impaired cell fusion and resulted in abnormally spread osteoclasts. The RhoGTPase Wrch1 (Wnt-1 responsive Cdc42 homolog) is a Cdc42- and Rac1-related protein whose expression is increased in Wnt-1 transformed mouse mammary epithelial cells (32). In contrast to most RhoGTPases, Wrch1 exhibits a rapid intrinsic guanine nucleotide exchange activity *in vitro* (33). This suggests that Wrch1 may not require an exchange factor to be active in the cell. The mRNA steady state level thus appears as an essential regulatory step of Wrch1 activity. Little is known about Wrch1 cellular functions so far. Wrch1, Cdc42 and Rac1 share some effectors such as PAK1, but unlike Cdc42 and Rac1, Wrch1 displays an atypical proline rich N-terminal extension that can bind to Nck1 and Grb2, two adaptor proteins involved in cell adhesion (33, 34). Since the over-expression of Wrch1 in fibroblastic cells affects the formation of focal adhesions (34), Wrch1 up-regulation

could be involved in the control of the formation of osteoclast specific adhesion structures, such as the podosomes and the sealing zone.

5 RhoGTPases were not found expressed in RAW264.7 cells: RhoH, Rac3, TCL, Rnd3 and RhoD. As far as RhoH is concerned, our results are in agreement with reports showing that while RhoH expression is restricted to hematopoietic tissues (35), it is expressed at very low levels in myeloid cells (36) from which the RAW264.7 cell line was derived.

Finally we found that the expression of 12 RhoGTPase genes was not affected by RANKL. Most of these RhoGTPases require a specific guanine nucleotide exchange factor to be activated and trigger downstream signaling pathways. We thus looked at the RhoGEF family and identified 42 RhoGEFs expressed in osteoclasts differentiated from RAW264.7 cells. Only Arhgef8/Net1 and Dock5 were up-regulated during osteoclastogenesis in RAW264.7, BMM and HSC cells.

Silencing of the RhoGEF Arhgef8 affected osteoclast differentiation and morphology, as also seen for the GTPase Wrch1. Arhgef8 is an exchange factor for RhoA. The regulation of Arhgef8 protein activity is well documented (37, 38), nevertheless the cellular functions of Arhgef8 remain unknown. Arhgef8 gene was recently found up-regulated in interleukin-2-stimulated human T-lymphocytes (39) and during CD40 ligand-induced maturation of monocyte-derived dendritic cell (40). In the body context, osteoclasts, dendritic cells and lymphocytes are highly migrating cells between and within anatomical compartments. Moreover, Arhgef8 was shown to regulate gastrulation movements during *Xenopus* development (41). Thus, Arhgef8 could be an important regulator of cell adhesion and migration through the activation of RhoA, a RhoGTPase with important functions in osteoclasts, dendritic cells and lymphocytes (42-44). In particular, RhoA is essential for osteoclast polarization and for the formation of the sealing zone (4). It would be interesting to explore the function of Arhgef8 in these processes.

Silencing of Dock5 induced cell death in RAW264.7 cells. Interestingly, survival was not affected in NIH3T3 fibroblastic cells, suggesting Dock5 has a specific function in monocytes and more specifically in osteoclasts, as we show that expression is up-regulated during osteoclastogenesis. Based on sequence homology, structural and phylogenic data, the RhoGEF Dock5 was classified with Dock2 in the Dock180 subfamily of CZH proteins. Proteins in this subgroup exhibit 50-65% amino acid sequence similarity. Dock180 and Dock2 are Rac1 exchange factors, suggesting that Dock5 could also activate this GTPase (10). Nevertheless, Dock5 has not been cloned yet and thus no functional data is available. Proteins from the Dock180 subgroup have a binding site for ELMO, an adaptor that targets Dock180 to the membrane where it activates Rac (45). Dock180 controls

integrin-stimulated cell migration and spreading as well as apoptotic cell engulfment. Dock180 is ubiquitous whereas Dock2 is restricted to the hematopoietic cell lineage and involved in various aspects of lymphocyte biology including homing, activation, adhesion and migration (10). Our study suggests that Dock5 may have specific functions in monocytes, in particular in osteoclasts.

In conclusion, we have performed a detailed analysis of RhoGTPase and RhoGEF expression during RANKL-induced osteoclast differentiation. We have identified three novel genes up-regulated by RANKL during osteoclastogenesis, using both *in vitro* and *ex vivo* osteoclast differentiation systems. Gene silencing confirmed the importance of these genes in osteoclasts. Given the importance of RhoGTPase signaling pathways in many aspects of osteoclast physiology, further functional studies of the RhoGEFs Dock5, Arhgef8 and of the RhoGTPase Wrch1 should shed new light on the mechanisms of osteoclast differentiation and bone resorption.

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## FIGURE LEGENDS

Figure 1: RhoGTPase and RhoGEF expression in RAW264.7 cells. All bar and line graphs (log scales) show the cDNA levels of the indicated genes relative to Gapdh in the same sample, determined by quantitative RT-PCR. Error bars: 95% confidence limits of the Gene/Gapdh ratios. All graphs are representative of 3 independent experiments. Figures indicate folds between RANKL-untreated (white bars) and -treated (dashed bars) cells, \*: significant changes. (A): Expression of Src and TRAP in RAW264.7 cells at days 0, 3, 6 and 8 of RANKL treatment. (B): Expression of RhoGTPases during osteoclastogenesis in RAW264.7 cells. Total RNAs from RAW264.7 cells were prepared as in (A). For space saving reasons, several RhoGTPases were pooled in the same graphs but their expression levels relative to Gapdh are not intended to be compared with each other. (C): Expression of Wrch1 and Cdc42 in RAW264.7 cells at days 0, 3, 6 and 8 of RANKL treatment. (D) RhoGEF expression in RAW264.7 cells after 6 days of Mock (white bars) or RANKL (dashed bars) treatment. (E): Levels of Arhgef8/Net1 protein in RAW264.7 cells. Total cell extracts were prepared from RAW264.7 cells treated as in (D) and western blot analysis was performed with the indicated antibodies.

Figure 2: RhoGTPase and RhoGEF expression in HSCs and BMMs. All bar graphs (log scales) show the cDNA levels of the indicated genes relative to Gapdh in the same sample, determined by quantitative RT-PCR. Error bars: 95% confidence limits of the Gene/Gapdh ratios. All graphs are representative of 3 independent experiments. Figures indicate folds between RANKL-untreated (white bars) and -treated (dashed bars) cells, \*: significant changes. For space saving reasons, several genes were pooled on the same graphs but their cDNA levels relative to Gapdh are not intended to be compared with each other. (A-B): Osteoclast differentiation from Lin-CD117+ HSCs. Purified HSCs, grown in the presence of RANKL and M-CSF for 6 days, were fixed and stained for actin and nuclei (A) or TRAP enzyme (B). (C) Expression of Src and TRAP. Total RNAs were prepared from HSCs after a 6 day treatment with M-CSF (white bars) or M-CSF+RANKL (dashed bars). (D-E): Expression of Wrch1 and Cdc42 during osteoclastogenesis in HSCs (D) and BMMs (E). Total RNAs were prepared from HSCs treated as in (C) and total RNAs from BMMs after 5 days of M-CSF (white bars) or M-CSF+RANKL (dashed bars) treatment. (F-G) Expression of RhoGEFs in M-CSF (white bars) or M-CSF+RANKL (dashed bars) -treated HSCs (F) and BMMs (G). Total RNAs were prepared from HSCs and BMMs treated as in (D-E).

Figure 3: Suppression of Wrch1, Arhgef8 and Dock5 gene expression in RAW264.7 cells by shRNAs. Bar graphs (log scales) in B, C, D and H show the cDNA levels of the indicated genes relative to Gapdh in the same sample, determined by quantitative RT-PCR. Error bars: 95% confidence limits of the Gene/Gapdh ratios. All

graphs are representative of 3 independent experiments. (A) Mouse shRNA target sequences. Target regions are numbered after their positions in the open reading frames. (B-C) Wrch1 (B) and Arhgef8 (C) silencing in RAW264.7 cells. Total RNAs were prepared from cells expressing the indicated shRNA and treated for 0 days (white bars), 3 days (dashed bars) or 5 days (squared bars) with RANKL. (D-H) Effect of Wrch1 and Arhgef8 silencing. RAW264.7 cells expressing the indicated shRNAs targeting Wrch1 (dashed bars), Arhgef 8 (dotted bars) or Luciferase (white bar) were grown for 5 days in the presence of RANKL, fixed and stained for TRAP and DNA. (D) For each shRNA, the mean fusion indexes of three independent experiments with 95% confidence limits determined by ANOVA are shown. Mean fusion indexes were significantly different from control Luciferase shRNA expressing cells (\*) and non significantly different between cells expressing shRNAs targeting the same gene (NS), with  $P < 0,05$ . (E-H) Representative TRAP staining of non infected control cells (E), and cells expressing the indicated shRNA (F-H). (I-K) Dock5 silencing in NIH3T3 cells. (I) Total RNAs were prepared from NIH3T3 cells expressing the indicated shRNA targeting Luciferase (white bar) or Dock5 (lined bars) after 5 days in growth medium. (J-K) Representative actin staining of the same cells expressing the indicated shRNA.

Table 1. Primers used in the real time PCR analyses.

Gene name	Accession no.	Upstream	Downstream	size
<b>Rho GTPases</b>				
Cdc42	NM_009861	tgacagactacgaccgctaa	caacaagcaagaaaggagtc	129
Rac1	NM_009007	aagagatcggtgctgtcaaa	ttacaacagcaggcatttctc	120
Rac2	NM_009008	gcctggcactggccaaggat	ggcgcttctgctgctgtgtg	118
Rac3	NM_133223	gacaaggatacattgaacg	tacagtgcacttctgctg	190
RhoA	NM_016802	ttcggaatgacgagcacacg	gtctagcttgcagagcagct	166
RhoB	NM_007483	cgccatggcggtgcgcatcc	tcatagcaccttgcagcagct	137
RhoC	NM_007484	ggcaagatgagcataaccagg	ccctccggcgcttattcttc	181
RhoD	NM_007485	cgcgctcccagggtgcgggag	gccccggctgtgtcccagatt	198
RhoE/Rnd3	NM_028810	catatgaccagggggcaaat	tgctaggcatgtgcgaaatc	175
RhoF/Rif	NM_175092	ccgagcactacgccccgctg	agcaccgtggggatccctcg	229
RhoG	NM_019566	gagtgcggtgtggtggcgca	acctctgggtgccatttgtg	154
RhoH	BC094937	gctgtgctggtaggggacagt	caggggcccggatacttctga	174
RhoJ/TCL	NM_023275	acaaccagctgcggccactc	tgtacagcagacgcgccaag	184
RhoN/Rnd2	NM_009708	ttctcccgatcttctgagc	ggccgtcccagcagttgagt	121
RhoQ/TC10	NM_145491	ggggcaagcagctacctttg	gcgcatattccttagctct	157
RhoU/Wrch1	NM_133955	gagaagccggtgcctgaaga	ctttggctgtagctgggagct	142
RhoV/Chp1	NM_145530	tccgactcacaacccccaa	gcggatcttctcagccagac	132
Rnd1	NM_172612	catcagccgtccagagacca	caccgctgctcgtaggaga	161
<b>Control Genes</b>				
Gapdh	M32599	acagtccatgccatcactgcc	gcctgcttcaccaccttctt	265
Src	NM_009271	gaactatgtgcaccgggacc	gagctcggtcagcagaatcc	225
Trap	AK008391	cacgatgccagcgacaagag	tgaccccgtatgtgctaac	465
<b>Rho GEFs</b>				
Arhgef12	NM_027144	ctccagccaccagcagcaac	ctgagccagccagcctttgg	253
Arhgef8	NM_019671	tgccaggctaaacgcttgc	tgaatgcagaaggcgaaccg	252
Dock5	XM_619261	tggtgacacagggacagtgg	cacccaactatgcacgtgg	239
Farp2	NM_145519	agccctgctgaggactagcc	gggcatgtgcgatgggaacc	165
Fgd6	NM_053072	agacgatgccactccacgc	ctcataacgactgtggcgcg	186
Geft	NM_028027	gccttgggtaggagccctg	ggggatcactgagagcctg	161
Itsn2	NM_011365	gactggtggcaaggagaaac	ccatgtaccgctcctctgtc	189
Vav3	NM_020505	ccaagagtccagcaaaccc	cagcaagctggatctttccc	186

Accession no., GenBank accession number.

Table 2. Expression of Rho GEFs in RANKL treated and control RAW264.7 cells.

Gene names	Probe Set	Accession no.	Call +RANKL	Call -RANKL
<b>Rho GEFs present in RANKL treated RAW264.7</b>				
Abr	1433477_at	NM_198018	P	P
Akap13/Lbc	1433722_at	XM_620601	P	P
Als2/Alsin	1417783_at	NM_028717	P	P
Arhgef1/p115Rhogef	1421164_a_at	NM_008488	P	P
Arhgef2/Gefh1/Lfc	1421042_at	NM_008487	P	P
Arhgef4/Asef	1435033_at	NM_183019	P	P
Arhgef6/aplhaPix/Cool2	1429012_at	NM_152801	P	P
Arhgef7/betaPix/Cool1	1449066_a_at	NM_017402	P	P
Arhgef8/Net1	1421321_a_at	NM_019671	P	P
Arhgef10	1452303_at	NM_172751	P	P
Arhgef11/PDZRhogef	1434926_at	NM_001003912	P	P
Arhgef12/LARG	1423902_s_at	NM_027144	P	P
Arhgef18/p114Rhogef	1418553_at	NM_133962	P	P
Dock1/Dock180	1443991_at	XM_194386	P	P
Dock2	1422808_s_at	NM_033374	P	P
Dock4	1436405_at	NM_172803	P	P
Dock5	1425747_at	XM_619261	P	P
Dock7/Zir2	1448892_at	NM_026082	P	P
Dock8/Zir3	1449419_at	NM_028785	P	P
Dock10/Zizimin3	1439247_at	XM_129913	P	P
Dock11/Zizimin2	1429028_at	NM_001009947	P	P
Ect2	1419513_a_at	NM_007900	P	P
Farp2/Fir/Frg	1440799_s_at	NM_145519	P	P
Fgd3	1450235_at	NM_015759	P	P
Fgd6/Etohd4	1419322_at	NM_053072	P	P
E130306D19/FLJ14642	1436725_at	XM_131404	P	P
Gef/p63Rhogef	1419978_s_at	NM_028027	P	P
Itsn1	1425899_a_at	NM_010587	P	P
Itsn2/Sh3d1B	1423184_at	NM_011365	P	P
Plekhg2/Clg	1418201_at	NM_138752	P	P
Plekhg5/Tech/Gef720/Syx	1452248_at	NM_001004156	P	A
Plekhg6	1425073_at	NM_198604	P	P
Prex1	1434069_at	XM_485102	P	P
Sgef	1429185_at	BB774721	P	P
Sos1	1421884_at	NM_009231	P	P
Sos2	1452281_at	XM_127051	P	P
Spata13	1437865_at	XM_147847	P	P
Tiam1	1418057_at	NM_009384	P	P
Trio/Tgat	1433745_at	BC051169	P	P
Vav1	1422932_a_at	NM_011691	P	P
Vav2	1435244_at	NM_009500	P	P
Vav3	1417123_at	NM_020505	P	P

P, present; A, Absent; Accession no., GenBank accession number; +RANKL, RAW264.7 cells treated with RANKL for 72 hours; -RANKL, control RAW264.7 cells; Probe set, Affymetrix coordinates.

Table 2. (Continued)

Gene names	Probe Set	Accession no.	Call +RANKL	Call -RANKL
<b>Rho GEFs absent in RANKL treated RAW264.7</b>				
9130221D24Rik/FLJ20184	1431629_at	NM_029953	A	A
Arhgef3/Xpln	1424250_a_at	NM_027871	A	P
Arhgef5/Tim	1452304_a_at	XM_133067	A	A
Arhgef9/Collybistin	1435974_at	XM_288123	A	A
Arhgef10l/Grinchgef	1460405_at	NM_172415	A	P
Arhgef14/Mcf21/Ost/Dbp	1434140_at	NM_178076	A	P
Arhgef15/VsmRhogef	1440358_at	NM_177566	A	A
Arhgef16/Neuroblastoma	1427386_at	XM_149562	A	A
Arhgef17/p164Rhogef	1433682_at	XM_133692	A	A
Arhgef19/Wgef	1437629_at	NM_172520	A	A
Bcr	1427265_at	XM_125706	A	A
Dbp/Mcf2	1419021_at	NM_133197	A	A
Depdc2/Prex2	1432047_at	XM_129358	A	A
Dnmbp/Tuba	1431244_s_at	NM_028029	A	A
Dock3/Moca	1428852_at	NM_153413	A	A
Dock6/Zir1	1427240_at	XM_486200	A	A
Dock9/Zizimin1	1444028_s_at	XM_358315	A	A
Farp1	1452280_at	BC030329	A	A
Fgd1	1416865_at	NM_008001	A	A
Fgd2	1419515_at	NM_013710	A	P
Fgd4/Frabin	1425037_at	NM_139232	A	P
Fgd5	1460578_at	NM_172731	A	A
Gm941	1458753_at	AK053944	A	A
Kalirin/Hapip/Duo/Duet	1436066_at	XM_488776	A	A
Ngef/Ephexin	1448978_at	NM_019867	A	A
Obscurin	1443632_at	NM_001003914	A	A
Plekhg1	1435363_at	XM_136911	A	A
Plekhg3	1440017_at	NM_153804	A	A
Plekhg4/Frabin	1457145_at	XM_620707	A	A
Rasgrf1	1422600_at	NM_011245	A	A
Rasgrf2	1421621_at	NM_009027	A	A
Rgnef/p190Rhogef	1419457_at	NM_012026	A	A
Scambio/Solo	1436768_x_at	NM_198249	A	A
Tiam2	1423186_at	NM_011878	A	A

P, present; A, Absent; Accession no., GenBank accession number; +RANKL, RAW264.7 cells treated with RANKL for 72 hours; -RANKL, control RAW264.7 cells; Probe set, Affymetrix coordinates.

Table 3. Selected control genes for gene array analyses.

Gene Name	Probe Set	Accession number	Ratio +/-RANKL
<b>A430-2 Affymetrix chip control genes</b>			
Cytoplasmic beta-actin	M12481_5_at	M12481	1.5
Cytoplasmic beta-actin	M12481_M_at	M12481	1.5
Cytoplasmic beta-actin	M12481_3_at	M12481	1.6
Glyceraldehyde-3-phosphate dehydrogenase	M32599_5_at	M32599	1.5
Glyceraldehyde-3-phosphate dehydrogenase	M32599_M_at	M32599	1.4
Glyceraldehyde-3-phosphate dehydrogenase	M32599_3_at	M32599	1.4
<b>RANKL regulated genes</b>			
Acid phosphatase 5, tartrate resistant	1431609_a_at	AK008391	475.5
Cathepsin K	1450652_at	NM_007802	104.2
DC-Specific transmembrane protein	1431970_at	AK014697	23.0
Integrin alpha V	1421198_at	NM_008402	9.7
Matrix metalloproteinase 9	1448291_at	NM_013599	6970.0
Nuclear factor of activated T-cells, cytoplasmic 1	1417621_at	NM_016791	15.3
Rous sarcoma oncogene	1450918_s_at	NM_009271	29.2
Wrch1	1449028_at	NM_133955	98.2

Ratio +/- RANKL, ratio between Affymetrix signals in RAW264.7 cells treated with RANKL for 72 hours and control RAW264.7 cells; Probe set, Affymetrix coordinates.

Table 4. Gene array and real-time PCR analyses of Rho GEF expression in response to RANKL in RAW264.7 cells.

Gene Name	Probe Set	Ratio +/-RANKL Affymetrix data	Ratio +/-RANKL Q-PCR Data
Dock5	1425747_at	13.0	23.5
Fgd6	1419322_at	7.6	18.8
Geft	1419978_s_at	6.8	8
Farp2	1440799_s_at	6.4	6.2
Vav3	1417123_at	4.4	4.8
Arhgef8	1421321_a_at	3.2	5.1
Arhgef12	1423902_s_at	3.1	4.5
Tiam1	1418057_at	2.9	ND
Abr	1433477_at	2.8	ND
Arhgef18	1418553_at	1.8	ND
Dock8	1449419_at	1.7	ND
Plekhg6	1425073_at	1.7	ND
Arhgef4	1435033_at	1.6	ND
Sos1	1421884_at	1.6	ND
Plekhg5	1452248_at	1.6	ND
Arhgef2	1427646_a_at	1.5	ND
Akap13	1433722_at	1.5	ND
Arhgef11	1434926_at	1.5	ND
Arhgef2	1421042_at	1.4	ND
Dock10	1439247_at	1.4	ND
Fgd3	1450235_at	1.3	ND
Arhgef1	1421164_a_at	1.3	ND
Sgef	1429185_at	1.3	ND
Als2	1417783_at	1.3	ND
Itsn2	1423184_at	1.3	1.2
Trio	1454711_at	1.2	ND
Prex1	1434069_at	1.2	ND
Arhgef10	1452303_at	1.1	ND
Trio	1433745_at	1.1	ND
Dock11	1429028_at	1.1	ND
Dock4	1436405_at	1.1	ND
Vav1	1422932_a_at	1.0	ND
Plekhg2	1418201_at	1.0	ND
Dock7	1448892_at	0.8	ND
Arhgef6	1429012_at	0.8	ND
Vav2	1435244_at	0.8	ND
Arhgef7	1449066_a_at	0.7	ND
Itsn1	1425899_a_at	0.6	ND
Dock1	1452220_at	0.5	ND
FLJ14642	1436725_at	0.4	ND
Spata13	1437865_at	0.3	ND
Dock2	1422808_s_at	0.3	ND
Ect2	1419513_a_at	0.3	ND

Ratio +/- RANKL, ratio between Affymetrix signals in RAW264.7 cells treated with RANKL and control RAW264.7 cells; Affymetrix data, gene array signal; Q-PCR data, real time PCR values from Figure 2. ND, not determined.





