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Brn3 Transcription Factors Control Terminal Osteoclastogenesis

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Abstract Osteoclastic bone resorption is a central mechanism in skeletal development, remodeling and pathology. RANKL is a mandatory factor controlling osteoclastogenesis; however, the underlying signaling pathways are only partially characterized. Using a screening array for the investigation of differential transcription factor activation, we identified activation of the Brn3 transcription factor family as a downstream event of RANKL signaling during terminal osteoclastogenesis. RANKL stimulation induces expression of Brn3a and b and maximal transcriptional activity of Brn3 family members concurrent with osteoclastic giant cell formation. Immunohistochemical analysis revealed both nuclear and cytoplasmic localization of Brn3a and b in mature osteoclasts. Functional inhibition of Brn3 transcription factors resulted in inhibition of pre-osteoclast fusion and reduction in bone resorbing activity of mature osteoclasts. Furthermore, we identified synaptotagmin-1, a regulator of membrane and vesicular fusion, as downstream target of Brn3 with a role in osteoclast function. We conclude that Brn-3 represents a novel molecular differentiation factor that controls osteoclast maturation and function, suggesting an important role in bone metabolism. J. Cell. Biochem. 102: 1–12, 2007.

Key words: bone; osteoclast; transcription factor; differentiation; RANKL

Bone is a dynamic organ that undergoes continuous remodeling directed by a balanced activity of osteoblasts (bone synthesis) and osteoclasts (bone resorption). Many adult osteopenic conditions such as osteoporosis, periodontitis, arthritis, and certain malignancies are attributable in part to excessive bone resorption by osteoclasts. Osteoclasts differentiate under the regulation of the TNFα family protein Receptor Activator of NF-κB Ligand (RANKL), which is expressed by osteoblasts and stromal cells following their activation by circulating hormones and locally produced cytokines [Galibert et al., 1998; Udagawa et al., 1999; Chambers, 2000; Teitelbaum, 2000]. The differentiation of pre-osteoclasts to mature osteoclasts following RANKL stimulation involves early activation of the transcription factors NF-κB and AP-1 [Grigoriadis et al., 1994; Franzoso et al., 1997; Xing et al., 2002]. Additional transcription factors are required for terminal differentiation and bone resorptive function (e.g., MITF) [Mansky et al., 2002]. However, a complete picture of the transcriptional control mechanisms that regulate osteoclast differentiation is lacking. The identification of those transcription factors and their gene targets will provide new insights into the molecular pathways governing the ability of cells to differentiate to the osteoclast lineage.

Through screening arrays of transcription factor activity in osteoclast precursor cells, we found that transcriptional activity of the Brn3 transcription factor family is enhanced in response to RANKL stimulation. Brn-3/POU-domain transcription factors have previously been shown to be crucial in regulating differentiation and maturation of neuronal cells. Three related proteins exist, Brn3a, Brn3b,

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and Brn3c (also known as Pou4f1, Pou4f2, Pou4f3 respectively), which are derived from distinct genes. Brn3 proteins share more than 95% identity within a bipartite DNA-binding POU domain, which consists of two highly conserved regions, connected by a variable linker of 14–26 amino acids. In vitro selection of an optimal DNA binding site had revealed a Brn3 consensus motif [(A/G)CTCATTAA-(T/C)] and similar sequences that are recognized by each of the Brn3 POU domains. Brn3 family members are expressed mainly in neurons of the trigeminal and dorsal root ganglia, retinal ganglion cells, cochlear hair cells, and several brainstem nuclei, both during development and in adulthood, but have not previously been identified in bone [Gerrero et al., 1993; Ninkina et al., 1993; Xiang et al., 1993, 1995, 1997a,b; Turner et al., 1994; Fedtsova and Turner, 1995; Ryan and Rosenfeld, 1997; McEvilly and Rosenfeld, 1999].

In the present studies, we investigated the role of the Brn3 transcription factor family in osteoclastogenesis and in regulating mature osteoclast function. As described herein, our data indicate that transcription factor family Brn3 is an important regulator during terminal differentiation of osteoclasts and regulates osteoclast function.

MATERIALS AND METHODS

Cell Culture

Mouse macrophage-like RAW 264.7 cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 1.5 g/L sodium bicarbonate and 10% fetal bovine serum (FBS, Invitrogen, CA). To induce osteoclast differentiation, recombinant mouse RANKL (R&D Systems, Minneapolis, MN) was added at a concentration of 10 ng/ml. In some experiments, normal mouse bone marrow cells were obtained from the femurs of 4-week old BALB/c mice, and were either co-cultured with calvarial cells isolated from 1-to 2-day old newborn mice for 10 days in αMEM/10% FBS supplemented with 10^{-8} M 1α,25-$(OH)_2$D3 (Biomol, Plymouth Meeting, PA) or cultured in the presence of MCSF (50 ng/ml) (R&D Systems) and RANKL (25 ng/ml) in αMEM/10% FBS to induce osteoclast formation.

Preparation of Nuclear Extracts

Cells were washed twice with PBS, pH 7.4, followed by suspension in 800 μl ice-cold lysis buffer (mmol/L: HEPES 10; KCl 10; EDTA 0.1; EGTA 0.1; DTT 1.0; PMSF 1.0; aprotinin 10 μg/ml, pepstatin 10 μg/ml, leupeptin 10 μg/ml). The collected samples were incubated on ice for 30 min, vortexed for 30 s after addition of 50 μl 10% Nonidet-P40, and centrifuged for 10 min at 4°C. The nuclei-containing pellets were suspended in ice-cold buffer (mmol/L: HEPES 20; NaCl 400; EDTA 1.0; EGTA 1.0; DTT 1.0; PMSF 1.0; aprotinin 10 μg/ml, pepstatin 10 μg/ml; leupeptin 10 μg/ml), incubated on ice for 2 h with frequent mixing, and centrifuged for 10 min at 4°C. The supernatants were collected as nuclear extract and stored at −70°C. The total protein concentration was determined using a protein assay kit (Pierce, Rockford, IL).

Screening of Transcriptional Activation

Nuclear extracts of cells at different times after RANKL stimulation (baseline, 30 min and 72 h) were incubated with biotin-labeled DNA-binding oligonucleotides (Panomics, Redwood City, CA). The protein/DNA complexes were separated from the free unbound probe by loading onto a 2% agarose gel in TBE buffer (M/l: TRIS1, boric acid 0.9, EDTA 0.01). After extraction of the protein/DNA complexes from the gel, the oligonuleotides were isolated following heating of the sample to 95°C for 3 min. The collected probes were hybridized overnight to a TranSignal Array membrane (Panomics, Redwood, CA) containing the consensus binding sequences for 54 different transcription factors. After reaction with streptavidin–alkaline phosphatase (Perkin Elmer, Wilmington, DE), the bound probes were visualized on a Kodak film by using CDP-Star chemiluminescence reagent (Tropix, Bedford, MA). Films were scanned and timepoints were normalized over baseline to determine changes in activity.

Electrophoretic Mobility Shift Assays (EMSA)

Brn3 binding studies were performed using double stranded oligonucleotides containing a Brn3 consensus site (5′-CACAGCTCATTAA-CGCGC-3′, 3′-GTGTCGAGTAATTGCGCG-5′) (Panomics, Redwood City, CA). The oligonucleotides were end labeled with ^{32}-ATP using T4 polynucleotide kinase (Promega, Madison, WI) and incubated with nuclear extract for 20 min at room temperature. The samples were loaded on a 4% non-denaturating polyacrylamide gel. After electrophoresis, the gel was dried and

exposed to Kodak film. Antibody supershift assays were carried out to confirm the identity of proteins in the Brn3 binding complex. Nuclear extracts were incubated with the appropriate antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C followed by the addition of the labeled oligonucleotide probe. Data from all experiments were expressed as a change in activity over baseline.

RT-PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA), reverse transcribed to cDNA with SuperScript II (Invitrogen), and used as a template for the PCR reaction. The PCR was done using following primers: brn3a sense: 5′GCCAACCTCAAGATCCCGGGCG′3; brn3a anti-sense: 5′CCAGTTTCTCGGCGATGGCGGC′3; brn3b sense: 5′CAC-GGTGGTGTCCACTCCGG′3; brn3b antisense: 5′CCGCGATCTTCTCCGAGGAG′3; synaptotagmin 1(Syt 1) sense: 5′-GACCGCTTCTCCAAGCACGAC-′3; Syt 1 anti-sense: 5′-CTGCGCCGGTGCTGTTGTAG-′3. Data from all experiments were expressed as a change in expression over baseline.

Western Blot Analysis

Cells were washed twice with PBS and lysed with buffer (mmol/L: Tris HCL 20; NaCl 150; EDTA 1; sodium orthovanadate 1; PMSF 0.5; 10 μg/ml aprotinin; 1% Nonidet p40; 0.1% SDS). The samples were incubated on ice for 1.5 h, and centrifuged for 10 min at 4°C in an Eppendorf centrifuge. The supernatants were collected and stored at −20°C. The protein concentration was determined using a protein assay reagent (Pierce). The samples were mixed with loading buffer (Sigma, St. Louis, MO), heated at 95°C for 5 min, electrophoresed on a 12% SDS–polyacrylamide gel, and proteins were transferred onto a PVDF membrane. The proteins were detected with a primary anti-Brn3a or anti-Brn3b antibody (Santa Cruz Biotechnology) using a Western blot kit (Invitrogen). Positive bands were visualized using the chemiluminescence reaction (Invitrogen) followed by exposure to photographic film. Data from all experiments were expressed as a change in protein expression over baseline.

Immunohistochemistry

Differentiated RAW264.7 cells and bone marrow cells from Balb/c mice were analyzed using a monoclonal antibody against Brn3a, a polyclonal antibody against Brn3b (Santa Cruz Biotechnology) and a polyclonal antibody against Syt1 (Abcam, UK). The complementary secondary antibodies were conjugated with Alexa 488, Alexa 648, or Rhodamine (Molecular Probes, Eugene, OR). Samples were analyzed on a Leica TCS SP2 confocal microscope.

Histological Analysis of Osteoclast Differentiation

Tartrate-resistant acid phosphatase (TRAP) was detected using a commercially available kit (Sigma-Aldrich, St. Louis, MO). In brief, after RANKL stimulation, RAW264.7, bone marrow cells or sections were washed twice with PBS (pH 7.4), fixed, washed again, and incubated with TRAP staining solution for 30 min at 37°C. TRAP-positive cells containing three or more nuclei were counted as osteoclasts. Images of the osteoclasts were taken under a bright field microscope and normalized onto a standardized quadrangle. The measurement of total osteoclast area was done using the Scion program (NCBI).

Analysis of Osteoclast Resorptive Activity

RAW264.7 or bone marrow cells in coculture with calvarial cells were induced to differentiate for 4–5 days or 7–10 days, respectively, on a 3D Collagen Cell Culture System matrix following the manufacturer's instructions (Chemicon International, Inc., Temecula, CA). Cells were removed by disolving the matrix in 0.2% collagenase, and were replated on 16-well Osteologic Multitest Slides (BD Bioscience, Bedford, MA) or 100 μm thick bovine bone slides. Cells were cultured for an additional 2 days in either DMEM/10%FBS with 10 ng/ml RANKL (RAW264.7), or αMEM/FBS with 25 ng/ml RANKL for osteclasts derived from bone marrow. Resorption lacunae were photographed by bright field (Osteologic) or electron microscopy (bone, dentin slides) and analyzed using an image analyzing software (Scion Image NCBI).

Inhibition of Transcriptional Activation of Brn3-Proteins

Double-stranded oligonucleotides were prepared from complementary single stranded phosphorothioate-bonded fluorescence-labeled oligonucleotides by melting at 95°C for 5 min

followed by a cooling phase at room temperature. The sequences of the oligonucleotides were as follows: Brn3 sense: 5′-CACAGCTCATTAACGCGC-′3; Brn3 anti-sense: 5′-GCGCGTTAATGAGCTGTG-′3; Brn3 mutated sense: 5′-CAGAGCTCAGCAACGCGC-′3′; Brn3 mutated anti-sense: 5′-GCGCGTTGCTGAGCTCTG-′3. All decoy oligonucleotides were transfected into the cells using Oligofectamine Reagent (Invitrogen) and efficiency of transfection was monitored by flourescence microscopy.

Inhibition of Syt-1 Expression

Silencing RNA against Syt-1 and non-specific control siRNA were purchased from Quiagen (Valencia, CA). Scrambeled non-specific RNA and siRNA were transfected into cells using Lipofectamine Reagent (Invitrogen) and efficiency of transfection was monitored by flourescence microscopy.

Statistical Analysis

Means were compared by using one-way analysis of Variance (ANOVA) or Student's *t*-test. $P \leq 0.05$ was considered as significant.

RESULTS

Screening of Transcriptional Activation in Osteoclast Differentiation

Incubation of RAW 264.7 cells with RANKL for 5 days resulted in the formation of multinucleated osteoclast-like cells. As previously shown, these cells express the osteoclast markers tartrate-resistant acid phosphatase (TRAP), cathepsin K, matrix metalloproteinase-9 (MMP-9), the proton pump protein Atp6i, and form resorption pits on bone slices and submicron calcium phosphate films [Battaglino et al., 2002].

A transcriptional screening analysis was performed on RAW 264.7 cells after RANKL stimulation using the TranSignal Screen (Panomics, Carlsbad, CA). The differential binding of nuclear proteins to 54 different transcription factor consensus sites was explored over a time course of 3 days and compared to non-stimulated cells. This analysis revealed a time-dependent binding of nuclear proteins to the Brn3 consensus site (Fig. 1A). In addition, NF-κB transcriptional activity increased after 30 min, and AP-1 and NFATc showed a delayed increase of binding activity in response to RANKL stimulation, consistent with previous findings of other investigations [Grigoriadis et al., 1994; Franzoso et al., 1997; Xing et al., 2002].

To verify these results, EMSA analysis was performed using specific Brn3 oligonucleotide probes. These experiments confirmed an increase in the formation of protein/DNA binding complexes beginning on day 1, and peaking on day 4 after RANKL stimulation (Fig. 1B).

Characterization of Brn3 Family Member Levels and Function During Osteoclastogenesis

The Brn3 transcription factor family consists of Brn3a, Brn3b, and Brn3c [Xiang et al., 1995]. EMSA supershift analysis showed activation of both Brn3a and Brn3b during RANKL-induced osteoclast differentiation (Fig. 1C). Brn3c could not be assessed due to a lack of available antibodies. To determine the effects of RANKL stimulation on Brn3 expression, RAW264.7 cells were stimulated with RANKL and gene induction and protein expression was determined using semi-quantitative RT-PCR and Western blotting, respectively. We found a strong increase in *brn3b* and *brn3a* mRNA levels that peaked on day 2 after RANKL stimulation (Fig. 2A). Western blot analysis revealed a delayed increase in Brn3b and Brn3a protein levels that peaked on day 3 (Fig. 2B); Brn3a was upregulated to a lower extent compared to Brn3b. Finally, immunohistochemical analysis by confocal microscopy demonstrated nuclear and cytoplasmic localization of Brn3b and Brn3a in RAW264.7 cells stimulated with RANKL for 4 days and osteoclasts derived from bone marrow cells (Fig. 2C). These data indicate that both Brn3a and Brn3b are activated and positively regulated during RANKL-induced osteoclastogenesis and detectable in mature osteoclasts in vitro suggesting a role for those factors in developing and active osteoclasts.

Inhibition of Protein/Brn3 Consensus Site Interaction by Decoy Oligonucleotides

To determine the impact of protein binding to the Brn3 consensus site, we inhibited this interaction using decoy oligonucleotides that contain a consensus Brn3 binding site and, therefore, can decrease DNA/protein complex formation via competitive inhibition. The transfection efficiency of FITC-labeled decoy oligonucleoties was approximately 80% as assessed

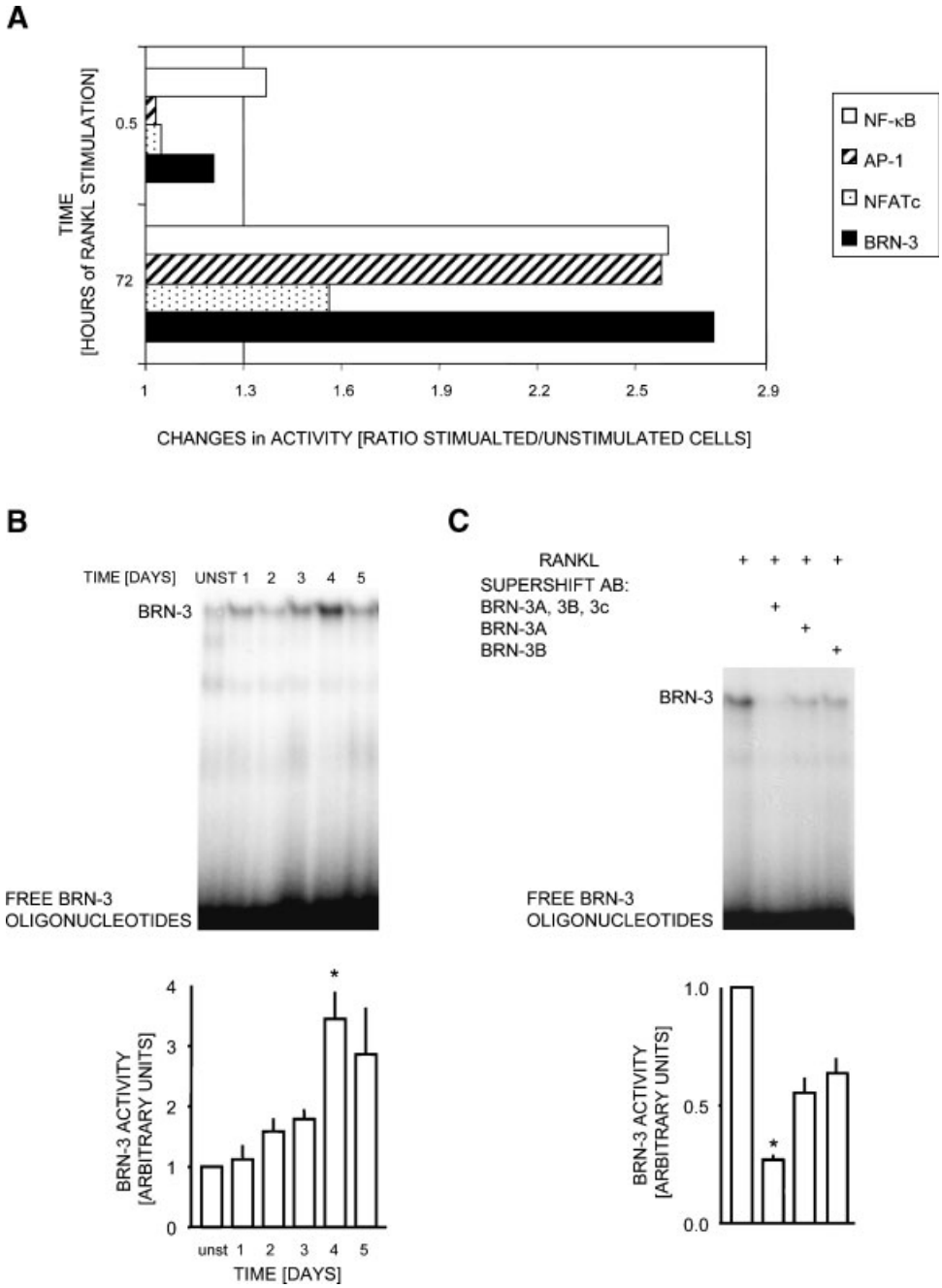

Fig. 1. RANKL-induced nuclear protein binding to the Brn3 consensus site. **A**: Transcriptional screening analysis performed on macrophage-like RAW264.Seven cells at 30 min and 3 days after RANKL stimulation showed a time-dependant activation of Brn3. The screening system was validated by the activation profiles of the transcription factors NF-κB, AP-1, and NFATc known to be activated in osteoclastogenesis. Activation ratios above 1.3 were considered as an increase in transcriptional activity compared to unstimulated cells. **B**: Time course of RANKL induced nuclear protein binding to the Brn3 consensus site; combined results of three separate experiments. $^*P \leq 0.01$, compared to non-stimulated cells. **C**: Proteins responsible for binding and activating the Brn3 consensus site and their RANKL-induced expression regulation. Supershift experiments showed the presence of Brn3a and Brn3b in the Brn3/DNA binding complex. Combined results of three separate experiments. $^*P \leq 0.05$, compared to day 4 nuclear extract.

by fluorescence microscopy (Fig. 3A). Transfected cells were extracted for their content of nuclear proteins and the residual Brn3 binding activity was analyzed by EMSA. Binding to the Brn3 consensus site was reduced by approximately 70% compared to non-transfected cells or cells transfected with mutant oligonucleotides (Fig. 3B). These data indicate

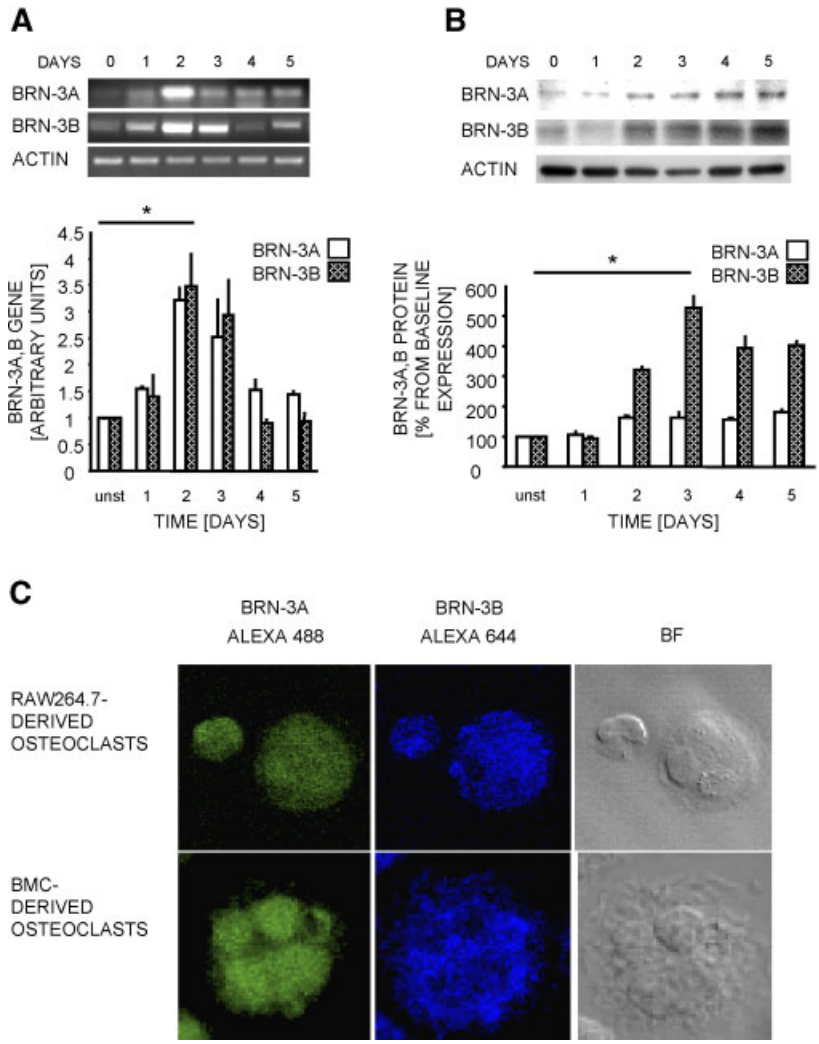

Fig. 2. **A**: Expression of *brn3b* and *brn3a* mRNA increases in response to RANKL stimulation of RAW264.7 cells. $^*P \leq 0.05$ versus baseline. **B**: Brn3b and Brn3a Western blot analysis revealed an increase in protein levels after stimulation with RANKL. Combined results of three separate experiments. $^*P \leq 0.05$ versus baseline. **C**: Immunfluorescence staining illustrates nuclear and cytoplasmic localization of Brn3a and Brn3b in RAW264.7-derived osteoclasts and bone marrow cell derived osteoclasts after 4 and 6 days in culture, respectively. BF, brightfield. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that transfection of RAW 264.7 cells with Brn3 decoy oligonucleotides results in specific inhibition of protein binding to the Brn3 consensus site in vitro.

Next, we investigated the effects of selective inhibiting Brn3 transcriptional activity during osteoclast formation. Cells were stimulated with RANKL, transfected on day 2, and monitored daily for RANKL-induced formation of osteoclastic giant cells derived from RAW264.7 cells. Transfection of Brn3 decoy or mutant oligonucleotides resulted in similar total numbers of TRAP-positive osteoclasts. However, cell fusion was inhibited by up to 32% on day 5 by Brn3 decoy oligonucleotides compared to controls, as assessed by the reduction in total osteoclast surface area (Fig. 3D). In contrast, FITC-labeled Brn3 mutant oligonucleotides were incorporated into cell nuclei without affecting cell fusion and differentiation. Notably, multinucleated cells that did form in the presence of Brn3 decoy oligonucleotide were consistently non-transfected whereas cells transfected with Brn3 decoy oligonucleotides remained smaller and less differentiated (Fig. 3C). Further analysis also demonstrated a significant reduction in the number of nuclei per cell versus cells transfected with Brn3

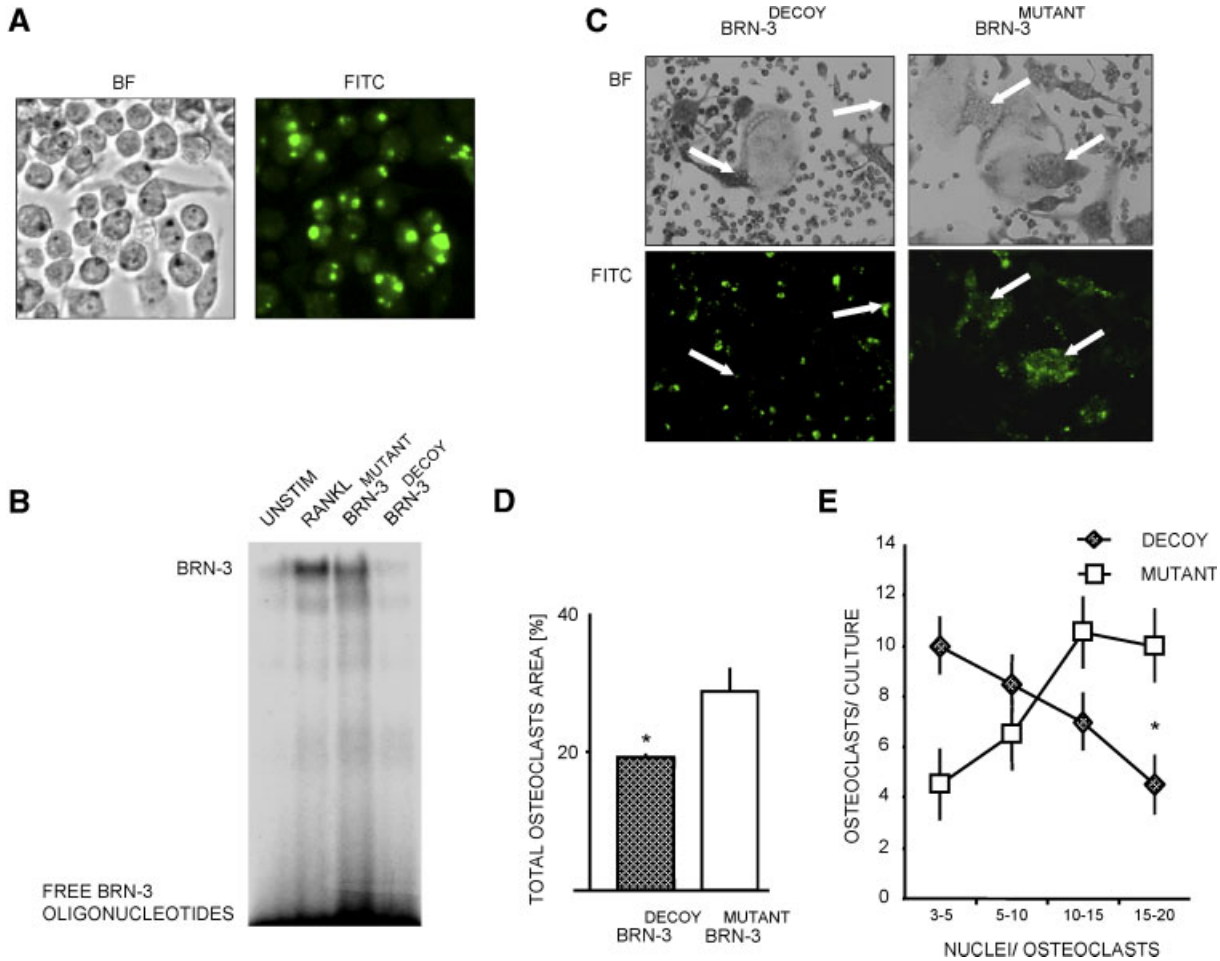

Fig. 3. Functional inhibition of protein binding to the Brn3 consensus motif by Brn3 decoy oligonucleotides. **A**: FITC labeled decoy and mutant oligonucleotides as a control were transfected into cells and transfection efficiency was monitored by fluorescence microscopy (FITC). BF, brightfield. **B**: Decoy oligonucleotides almost completely abolished RANKL-induced Brn3 binding activity in nuclear extracts, compared to mutant transfected control cells. **C**: Reduction of giant cell formation by functional inhibition of Brn3; brightfield (BF) and fluorescence microscopy (FITC) of RANKL-induced RAW264.7 cells transfected with FITC-labeled oligonucleotides. As indicated by arrows, FITC-labeled Brn3 mutant oligonucleotides transfected into cell nuclei did not interfere with giant cell formation. In the Brn3 decoy oligonucleotide cultures, multinucleated giant cells appear almost non-transfected compared to cells that integrated Brn3 decoy oligonucleotides and therefore remained smaller and less differentiated. **D**: Functional inhibition of binding to the Brn3 site by using decoy oligonucleotides resulted in a 32% reduction of giant cell formation assessed by total osteoclast surface area. $^{*}P \leq 0.05$ versus mutant transfected cells. **E**: Effect of functional inhibition of Brn3 on multinuclearity of osteoclasts. $^{*}P \leq 0.05$ versus mutant transfected cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mutant oligonucleotides (Fig. 3E). Taken together, these results indicate that protein binding to the Brn3 consensus site regulates late events in osteoclast differentiation, in particular terminal cell fusion processes.

Effect of Functional Inhibition of Brn3 on Osteoclast Resorptive Activity

The impact of protein/Brn3 consensus site interaction on RANKL induced osteoclast resorptive activity was also analyzed. Differentiated RAW264.7 cells, seeded onto Osteologic^{TM} slides and transfected with decoy oligonucleotides, exhibited 80% reduction of resorption activity in the decoy transfected cells compared to mutant oligonucleotide controls (Fig. 4A). This result was confirmed using bovine bone slices. Functional inhibition of protein binding to the Brn3 site by decoy oligonucleotides resulted in greater than 80% reduction of the number of resorption pits compared to mutant control transfected cells (Fig. 4C). We also generated osteoclasts by co-culture of normal bone marrow cells with calvarial cells in the

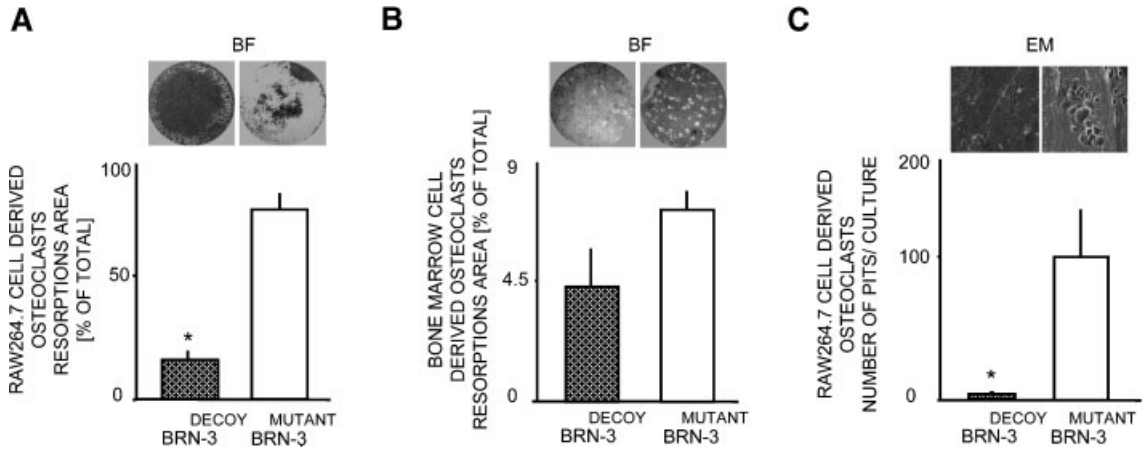

Fig. 4. Functional inhibition of Brn3 reduces RANKL-induced osteoclastic resorption activity. **A**: RANKL-induced osteoclastic resorption activity is diminished in differentiated RAW264.7 cells transfected with decoy oligonucleotides. Resorption was assessed on submicron calcium phosphate slides. BF, brightfield; $^{*}P \leq 0.01$ versus mutant transfected cells. **B**: RANKL-induced osteoclasts derived from normal bone marrow cells. Resorption was assessed on submicron calcium phosphate slides. BF, brightfield. **C**: RANKL-induced osteoclasts derived from RAW264.7 cells exhibited greater than 80% reduction in pit formation on bovine bone slides by functional inhibition of Brn3; scanning electron microscopy. EM, electron microscopy; $^{*}P \leq 0.05$ versus mutant transfected cells.

presence of 10^{-8}M 1α, 25-$(OH)_2D_3$ for 7 days. After transfer of osteoclasts to Osteologic™ slides, cells were transfected with decoy or mutant oligonucleotides to block protein/Brn3 site interaction. Similar to findings with RAW264.7 cells, a 41% reduction in osteoclastic resorptive activity was seen following functional inhibition of protein binding to the Brn3 site (Fig. 4B). Taken together, the above observations define protein binding to the Brn3 consensus site as an important event in the terminal stages of osteoclast differentiation and activation processes in response to RANKL stimulation.

Role of Syt1 as a Downstream Mediator of Brn3 Effects

Regulatory mechanisms and target genes involved in Brn3 controlled transcriptional events in osteoclasts are still undefined. We used the previously established Brn3 decoy oligonucleotide approach to specifically inhibit Brn3 transcriptional activation and to identify genes that are regulated via this interaction. RAW264.7 cells, transfected with decoy oligonucleotides or mutant controls, were differentiated in the presence of RANKL to mature osteoclasts. Cathepsin K and TRAP, two osteoclast-specific markers, revealed no difference between decoy and mutant transfected cells (data not shown). Notably, RANKL stimulation of pre-osteoclasts results in a robust induction of Syt-1 (Fig. 5A) which has previously been described as a target gene of the Brn3 transcription factor family in neuronal cells [Latchman, 1999]. Functional inhibition of Brn3 DNA-binding activity resulted in an 80% decrease in Syt1 expression. Syt1 has been classified as part of the SNARE complex that regulates vesicle to membrane fusion and exocytosis [Koh and Bellen, 2003]. Confocal analysis demonstrated a cytoplasmic localization of Syt1 in RANKL-induced osteoclasts derived from either RAW264.7 or mice bone marrow (Fig. 5B).

To further study the impact of Syt1 on osteoclast differentiation and activation, silencing RNA (siRNA) against Syt1 was transfected into bone marrow cell-derived osteoclast precursor cells (Fig. 5C). Reduction of Syt1 mRNA levels by siRNA resulted in a 62% decrease in bone resorbing activity of mature osteoclasts (Fig. 5D). These data indicate that Brn3 regulates the biological function of osteoclasts in part via Syt1, suggesting an important role for this gene in osteoclast function.

DISCUSSION

Mature, multinucleated osteoclasts derive from the monocyte/macrophage lineage in a differentiation process that involves the fusion of precursor cells to form multinucleated giant cells. As a mandatory inducing factor, RANKL activates precursor cells to develop into bone

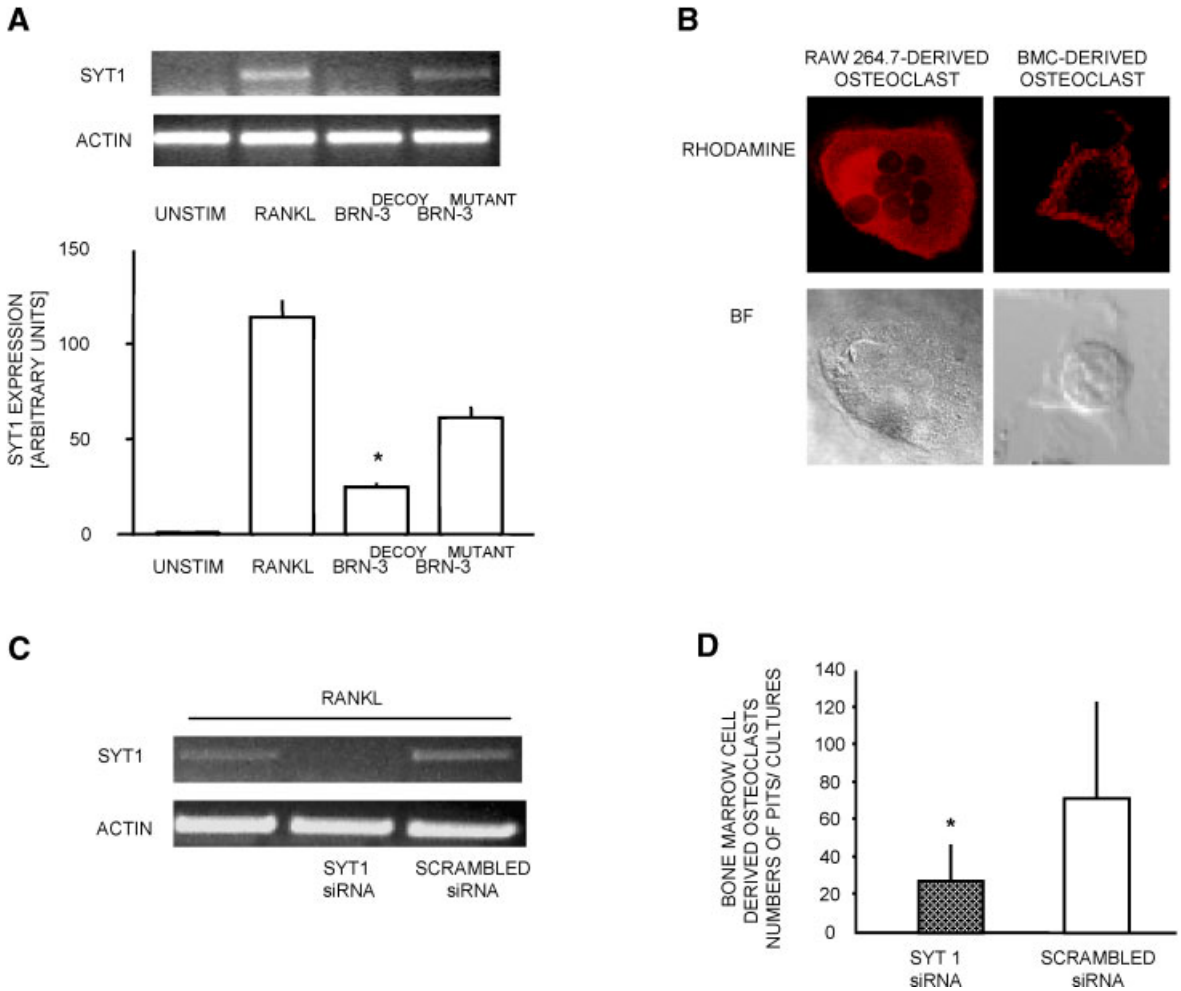

Fig. 5. Synaptotagmin, a Brn3 target gene, is necessary for bone resorption. **A**: Using decoy oligonucleotides in RAW264.7-derived osteoclasts, RANKL-stimulated expression of synaptotagmin 1 (Syt 1) was inhibited by functional blockade of the Brn3 consensus site. $^*P \leq 0.05$ versus mutant transfected cells. **B**: Immunfluorescence staining illustrates cytoplasmic localization of Syt1 in osteoclasts derived from RAW264.7 cells and bone marrow. BF, brightfield. **C**: SiRNA transfected into bone marrow derived osteoclasts reduces Syt1 expression as compared to scrambled control siRNA. **D**: Diminished expression of Syt1 reduces osteoclast activity, as determined by reduced pit formation on bovine bone slides as assessed by scanning electron microscopy. $^*P \leq 0.05$ versus baseline. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

resorbing cells. Differentiation results in profound morphological changes accompanied by a restricted gene expression pattern [Galibert et al., 1998; Hayashi et al., 1998; Chambers, 2000; Teitelbaum, 2000; Udagawa et al., 1999]. The regulation of osteoclast differentiation is driven by a complex network of transcription factors, which includes NF-κB, AP-1, NFATc, E-box factor MITF, and others [Grigoriadis et al., 1994; Franzoso et al., 1997; Mansky et al., 2002; Takayanagi et al., 2002; Xing et al., 2002].

In the present study, we systematically analyzed the transcriptional regulation of macrophage-like cells undergoing osteoclast differentiation using an activated transcription factor screening array approach. One of the most prominently activated transcription factor was Brn3, which peaked on day 4 after stimulation. Analysis in more detail revealed a continually increasing activity of Brn3a and Brn3b peaking at day 4, concurrent with detection of the first fused multinucleated osteoclasts. In addition to the transcriptional activity, Brn3a and Brn3b gene and protein expression is regulated in response to RANKL stimulation of pre-osteoclasts. To analyze the impact of Brn3 protein binding on osteoclast differentiation and activity, a decoy oligonucleotide approach was chosen which was able to simultaneously

inhibit the transcriptional binding of the Brn family members to this site. The suppression of potential protein/DNA binding activity in pre-osteoclasts resulted in a reduction of macrophage-derived multinucleated giant cell formation [Gruber et al., 1997]. The cells underwent morphological changes toward mature osteoclasts and expressed normal levels of TRAP and cathepsin K (data not shown), but demonstrated a significant reduction in multinuclearity. This finding, along with the kinetics of Brn3a and Brn3b/DNA binding activity, indicates an involvement of these proteins in late osteoclast differentiation including terminal cell fusion processes. In addition, functional inhibition of protein binding to the Brn3 consensus motif in differentiated macrophage-like RAW264.7 cells and in osteoclasts derived from co-cultured bone marrow cells decreased their bone resorptive activity dramatically.

Brn3 transcription factors were first described in neurons undergoing differentiation, in which there is a clearly distinctive but overlapping pattern of *brn3a, brn3b, brn3c* gene expression, as well as in some non-neuronal cells such as in testis during germ cell development [Ryan and Rosenfeld, 1997; Xiang et al., 1997b; McEvilly and Rosenfeld, 1999; Budhram-Mahadeo et al., 2001]. Interestingly, the phenotypes exhibited by Brn3 null mice reflect the temporal expression patterns of the three POU4 transcription factors. In dorsal root and trigeminal ganglia, *brn3a* is the first of the Brn3 family members that is expressed; gene deletion in vivo causes impairment of somatosensory and motor control in large part secondary to selective neuronal destruction in these regions. *Brn3b* is the first *brn3* gene to be detected in the developing retina, followed by *brn3a* and *brn3c* expression. Brn-3b null mice show severe defects in the retina and are missing about 70% of their retinal ganglion cells. In cochlear and vestibular hair cells of the inner ear, *brn3c* is expressed first. Its absence leads to hair cell degeneration with secondary loss of spiral and vestibular ganglion neurons [Gan et al., 1996; McEvilly et al., 1996; Mengqing Xiang et al., 1996; Xiang et al., 1997a,b, 1998; Xiang, 1998; Budhram-Mahadeo et al., 2001].

However, studies in chick ganglion cells indicate a functional redundancy of all Brn3 family members since all showed a similar ability to promote cell differentiation and development when ectopically expressed in retinal progenitor cells [Liu et al., 2000]. Brn3a and Brn3b both function as transcriptional activators with no observed inhibitory effect of Brn3b on the transactivational activity of Brn3a, in contrast to earlier studies that described antagonistic effects of these factors [Morris et al., 1994; Budhram-Mahadeo et al., 1999]. In addition, Brn-3b/3c double knockout mice exhibit an enhancement of severe retinal ganglion cell defects compared to targeted deletion of Brn3b alone [Wang et al., 2002]. Pan et al. [2005] revealed an equal role for Brn3 family members in retinal neurogenesis by restoring Brn3b function through placing Brn3a in its locus. This resulted in restoration of normal development and survival of retinal cells. Altogether, our findings suggest that the transcription factors Brn3a and b are activated during osteoclast differentiation and might play an important role in osteoclastogenesis and normal bone morphogenesis.

Given the foregoing, it appears that this mechanism is downstream of early-activated transcription factors such as NF-κB and AP-1, as well as another RANKL-induced intermediate-late transcription factor, MITF. In contrast to Brn3, MITF deficiency results in reduced expression of both TRAP and cathepsin K [Motyckova et al., 2001; Weilbaecher et al., 2001]. However, detailed mechanisms underlying activation of protein binding to the Brn3 consensus site are still unclear and require further study. We had used the decoy approach to identify potential Brn3 target genes in vitro. Our data indicate that synaptotagmin 1 (Syt 1) is part of a signaling cascade that is downstream of Brn3 activation during osteoclastogenesis. Although Syt 1 was originally described in neuronal cells, nothing was known about its regulation in osteoclastogenesis. We found Syt1 to be associated with osteoclast bone resorptive activity, which requires secretion of proteins such as cathepsin K and other enzymes into the extracellular space through the ruffled border. This finding is in line with its described function of regulating fusion inbetween vesicles and the outer cell membrane, thereby controlling secretion of proteins into the synaptic gap. Moreover, it describes an additional similarity inbetween osteoclasts and neurons. Despite different functions, both neurons and osteoclasts are electrogenic cells, which function in part through membrane depolarization [Teitelbaum, 2000]. Since Syt1 seems to be involved in osteoclast

activity only, further studies will be needed to clarify additional genes and mechanisms involved in Brn3-regulated osteoclastogenesis.

In conclusion, Brn3 DNA binding activity is enhanced in RANKL-induced osteoclast differentiation, and its inhibition results in suppression of giant cell formation and a reduction in osteoclast resorptive activity. Brn3a and Brn3b transcription factors might, therefore, serve as a promising new target for the modulation of bone mass, and the treatment of bone diseases caused by excessive bone resorption.

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