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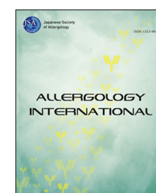

Original article

Involvement of PU.1 in NFATc1 promoter function in osteoclast development

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BM	bone marrow
ChIP	chromatin immunoprecipitation
DC	dendritic cells
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
TRAP	tartrate resistant acid phosphatase

A B S T R A C T

Background: The transcription factors NFATc1 and PU.1 play important roles in osteoclast development. NFATc1 and PU.1 transactivate osteoclast-specific gene expression and a deficiency in NFATc1 or PU.1 genes causes osteopetrosis due to an insufficient development of osteoclasts. However, the existence of cross-regulation between NFATc1 and PU.1 is largely unknown. In the present study, the role of PU.1 in NFATc1 expression was investigated.

Methods: Osteoclasts were generated from mouse bone marrow cells. PU.1 knockdown was performed with siRNA introduction. The mRNA levels in siRNA-introduced cells were determined by quantitative RT-PCR. The involvement of PU.1 in the NFATc1 promoter was analyzed by using a chromatin immunoprecipitation (ChIP) assay and a reporter assay. Retrovirus vector was used for enforced expression of PU.1.

Results: Introduction of PU.1 siRNA into bone marrow-derived osteoclasts resulted in a decrease in NFATc1 mRNA level. A ChIP assay showed that PU.1 bound to the NFATc1 promoter in osteoclasts. NFATc1 promoter activity was reduced in PU.1 knockdown cells as assessed by a reporter assay. PU.1 siRNA introduction also downregulated the expression of osteoclast-specific genes and tartrate resistant acid phosphatase (TRAP) activity. Enforced expression of PU.1 using a retrovirus vector increased NFATc1 expression and TRAP activity. When NFATc1 expression was knocked down by using siRNA, the induction of osteoclast-specific genes and TRAP-positive cells was suppressed without affecting the expression level of PU.1.

Conclusions: These results indicate that PU.1 is involved in osteoclast development by transactivating NFATc1 expression via direct binding to the NFATc1 promoter.

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Introduction

The transcription factor NFATc1 is a master regulator for the development and specific gene expression of osteoclasts. The Ets-family transcription factor PU.1, which plays important roles in the development of hematopoietic lineages, especially the monocyte/dendritic cell-lineage, is involved in osteoclast-specific gene expression with NFATc1.[1–3] The requirement for NFATc1 and PU.1 for osteoclast development has been observed in *in vitro* experiments. However, little is known about their roles *in vivo* in the function of osteoclastogenesis, because gene-targeted disruption of NFATc1 results in embryonic lethality[4]; moreover, PU.1 knockout mice are embryonic lethal[5] or die of severe septicemia within 48 h after birth,[6] and these mice also exhibit osteopetrosis.[7] It is largely unknown if PU.1 plays a role in NFATc1 expression, although it has been frequently observed that NFATc1 and PU.1 cooperatively transactivate the promoters of cell-type specific genes in osteoclasts. In the present study, considering that osteoclasts are myeloid organ and develop from a monocytic precursor and that PU.1 is important for the development of the monocyte/dendritic cell

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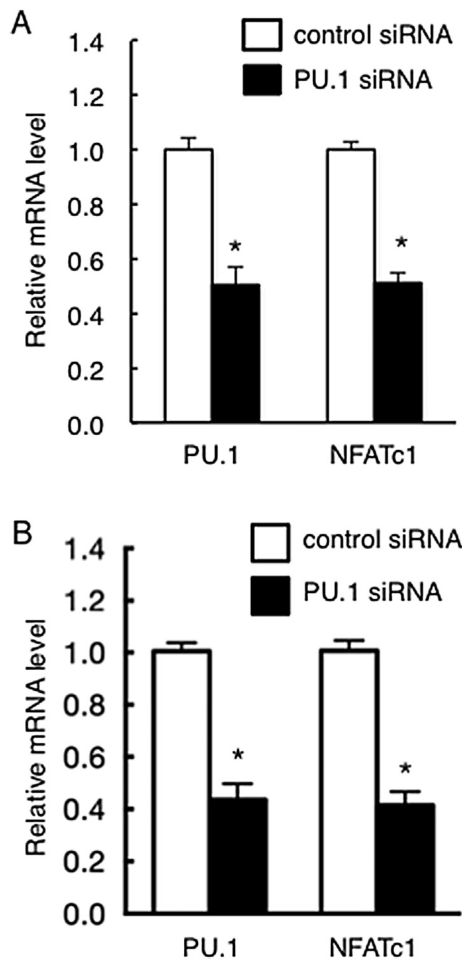

Fig. 1. Effect of PU.1 siRNA on NFATc1 mRNA level in osteoclasts. PU.1 siRNA or control siRNA was introduced into BM-derived osteoclasts on day 0 and day +3 by using lipofectamine (A) or on day 0 by electroporation (B), and the cells were harvested on day +4 for analysis of mRNA levels. The mRNA expression level of PU.1 and NFATc1 in the cells is displayed as the ratio of mRNA levels versus those in control siRNA-introduced cells. Data represent means ± SEM of four (A) or three (B) independent experiments. $^{*}p < 0.05$.

lineage, we hypothesized and investigated the possibility that PU.1 regulates the NFATc1 promoter in osteoclasts.

Methods

Mice

BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were approved by the Juntendo University Animal Experimental Ethics Committee and by the Tokyo University of Science Animal Experimental Ethics Committee and complied with National Institutes of Health guidelines for animal care.

Cells

Whole bone marrow (BM) cells from BALB/c mice were grown in α-MEM medium (GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS (Sigma–Aldrich, St. Louis, MO, USA), 100 μM 2-mercaptoethanol, 10 μM MEM nonessential amino acids (Sigma–Aldrich), 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 ng/ml recombinant murine (rm) M-CSF (PeproTech, London, United Kingdom) for 3 days (from day −3 to day 0). The resulting adherent osteoclast precursors were cultured in the presence of 50 ng/ml rmM-CSF and 100 ng/ml recombinant human (rh) RANKL (PeproTech) for an additional 3–8 days (from day 0 to day+3 ~ day +8). The mouse monocytic cell line RAW264.7 was maintained in RPMI 1640 medium (Sigma–Aldrich) supplemented with 10% heat-inactivated FBS and penicillin/streptomycin.

Knockdown of PU.1 expression by siRNA

PU.1 siRNA (Stealth Select RNAi, Sfpi1-MSS247676), NFATc1 siRNA [Stealth Select RNAi, Nfatc1-MSS275981 (#1) and Nfatc1-MSS275982 (#2)] and control siRNA (Stealth Negative Universal Control, no. 45-2001) were purchased from Invitrogen (Carlsbad, CA, USA). A 5-μl of aliquot of 20 μM siRNA was introduced into BM-derived developing osteoclast cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Electroporation was also used to introduce siRNAs into osteoclast cells as follows. A 2.5-μl of aliquot of 20 μM siRNA was introduced into 1×10^6 cells using a Mouse Macrophage Nucleofector kit (Lonza, Basel, Switzerland) with a Nucleofector II (Lonza) set at Y-001.

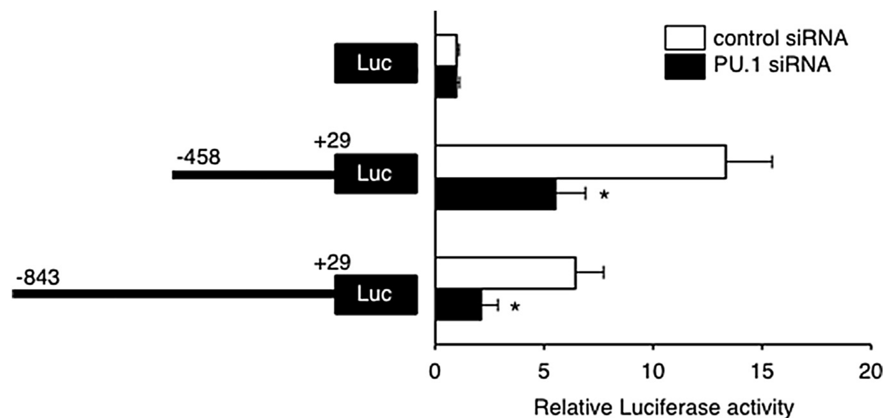

Fig. 2. Effect of PU.1 siRNA on NFATc1 promoter activity. A luciferase-linked NFATc1 promoter-driven pGL4 plasmid was transfected into RAW264.7 cells. PU.1 siRNA or control siRNA was introduced into the cells with reporter plasmids and luciferase activity was assayed. Relative luciferase activity is displayed as the ratio of luciferase activity versus that seen in cells transfected with the control pGL4-Basic plasmid. Data represent means ± SEM of three independent experiments. $^{*}p < 0.05$.

Quantification of mRNA by means of real-time PCR

Total RNA prepared from cells with an RNeasy kit (QIAGEN, Hilden, Germany) was reverse transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The mRNA level of PU.1, NFATc1, Ctsk, Oscar, Acp5, Itgb3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified by using a Step-One Real-Time PCR system (Applied Biosystems) with TaqMan Gene Expression Assays (Applied Biosystems: no. Mm01270606_m1 for PU.1, Mm00479445_m1 for NFATc1, Mm00484039_m1 for Ctsk, Mm00558665_m1 for Oscar, Mm00475698_m1 for Acp5, Mm00443980_m1 for Itgb3, and mouse GAPDH no. 44352339) and TaqMan Universal Master Mix (Applied Biosystems). The expression level of each mRNA was evaluated relative to that of GAPDH as described previously.[8,9]

Luciferase reporter assay

To generate reporter plasmids, 5'-flanking regions of the mouse *NFATc1* gene (−843/+29, or −458/+29) were introduced into the pGL4-Basic vector (Promega, Madison, WI, USA) by using PCR and

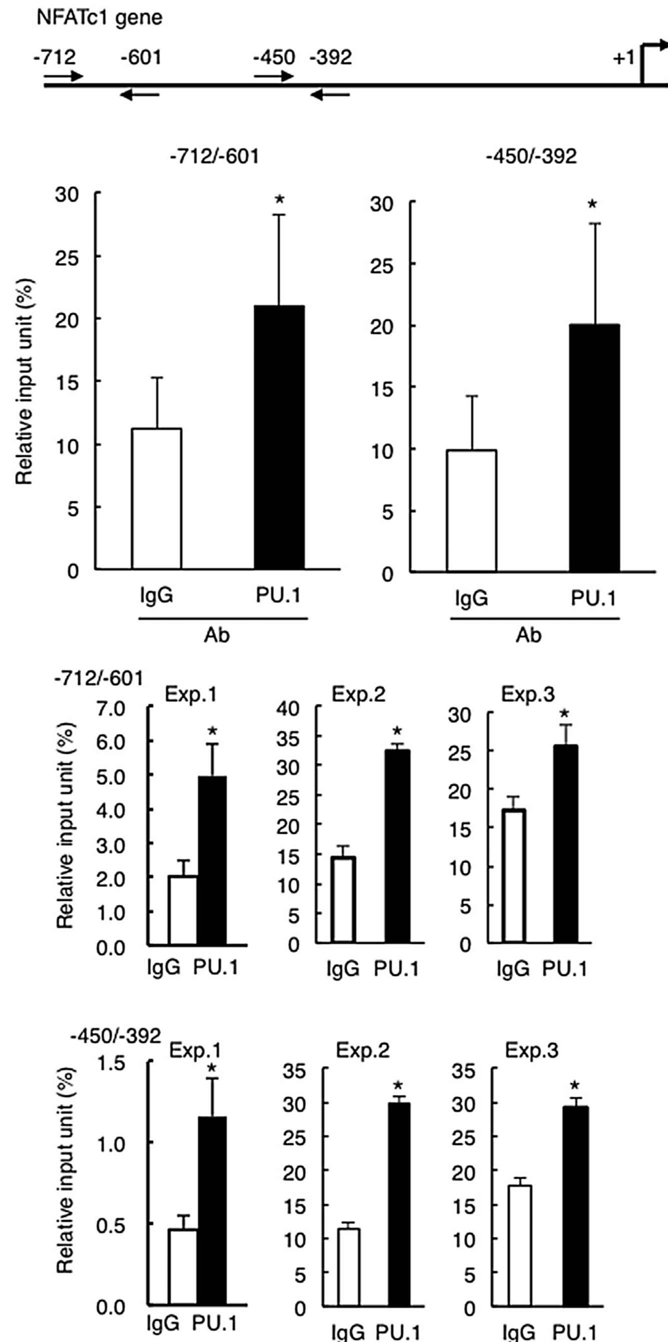

Fig. 3. PU.1 binds to the NFATc1 promoter in osteoclasts. Chromosomal DNA of BM-derived osteoclasts (day +8) was immunoprecipitated with control IgG (open bars) or anti-PU.1 Ab (closed bars) and analyzed for the NFATc1 promoter using quantitative real-time PCR. The results are expressed as means ± SEM of three independent experiments (top). The results of three experiments performed triplicate samples were also shown (bottom). $^{*}p < 0.05$.

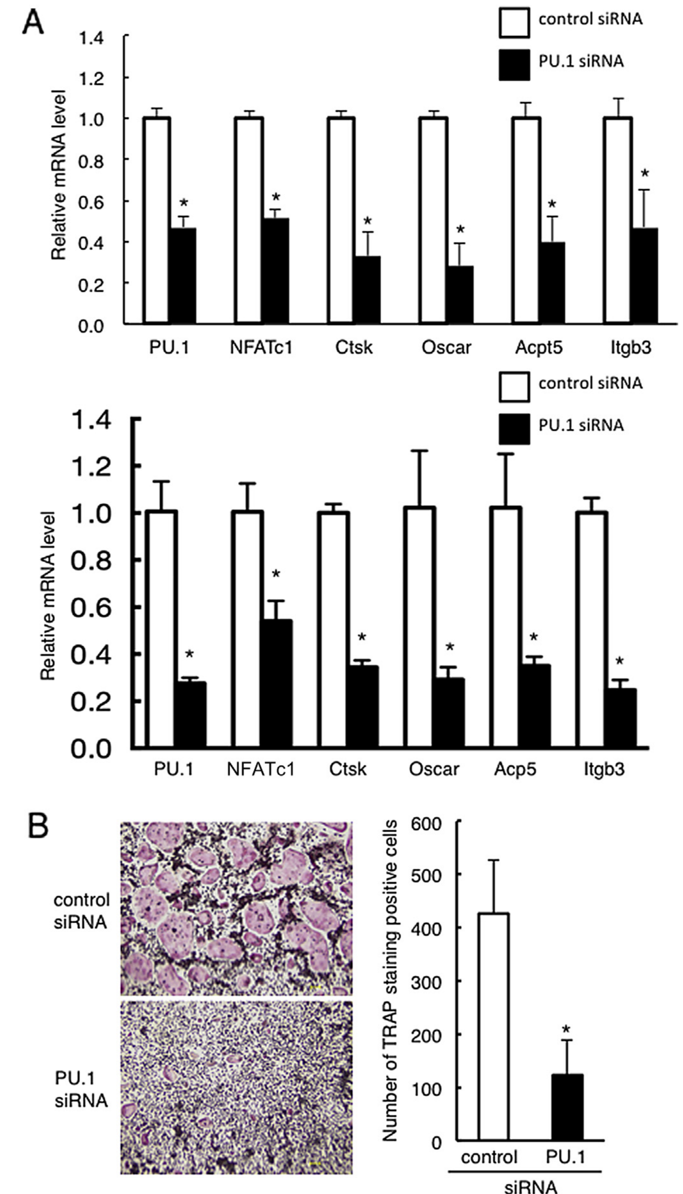

Fig. 4. Effect of PU.1 siRNA on osteoclast-specific gene expression and function. PU.1 siRNA or control siRNA was introduced into BM-derived cells on day 0, and day +3 by using the lipofectamine method (A top), or on day 0 by electroporation (A bottom), and cells were harvested on day +8 for analysis of mRNA levels (A) or for TRAP staining (B). The mRNA expression level of PU.1, NFATc1, Ctsk, Oscar, Acp5, and Itgb3 in BM-derived osteoclasts was analyzed using quantitative RT-PCR and is displayed as the ratio of mRNA levels versus those seen in control siRNA-introduced cells. Data represent means ± SEM of three independent experiments (A) or means ± SD of three independent experiments (B). $^{*}p < 0.05$.

site-directed mutagenesis as described previously.[10] The nucleotide sequences of synthesized oligonucleotides that were used as primers are listed in Supplementary Table 1. Transfection of RAW264.7 cells and determination of luciferase activity were performed as described previously.[8,10]

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously using the BM-derived osteoclasts on day 8.[8,11] Anti-PU.1 goat IgG (D-19, no. sc-5949, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat IgG (no. 02-6202, Invitrogen) were used. The amount of chromosomal DNA including the NFATc1 promoter was determined by the quantitative real-time PCR system using the primers listed in Supplementary Table 2. The ratio of immunoprecipitated DNA was calculated as described previously.[10]

Tartrate resistant acid phosphatase (TRAP) staining

TRAP activity of osteoclasts was determined as follows. Cells cultured in 24 wells-plates were fixed with 500 μl of 10% formaldehyde/PBS for 10 min after washing with PBS, and were then, rinsed with acetone/ethanol (1:1) for 0.5–1 min. After drying, the cells were incubated in 200 μl of TRAP solution (30 mg of the Dye Fast red violet LB salt, Sigma–Aldrich) that was dissolved in 50 ml of buffer (50 nM sodium tartrate, 0.1 M sodium acetate, pH 5.0) containing 5 mg naphthol AS-MX phosphate (Sigma–Aldrich) pre-dissolved in 0.5 ml formamide) for 10 min and washed with distilled water.

Introduction of PU.1 cDNA into osteoclast cells using a retrovirus vector

Retroviral infection of BM-osteoclasts was performed according to a previously reported method.[12] Full-length mouse PU.1 cDNA was amplified by PCR using following oligonucleotides as primers: PU.1 *Eco*RI-forward; 5'-aggagaattcgttacaggcgtgcaaaatgg-3′, and PU.1 *Sal*I-reverse; 5′-catggtcgactcagtggggcgggaggcgcc-3′ (the recognition sequences of *Eco*RI and *Sal*I are shown in italic, and the initiation and termination codons are underlined) and Balb/c mouse BM-derived dendritic cell cDNA as a template. The amplified cDNA was inserted into the *Eco*RI/*Sal*I site of p3xFlagCMV7.1 (Invitrogen). After confirmation of the nucleotide sequence, the *Bgl*II/*Sal*I DNA fragment encoding mouse PU.1 tagged with 3 Flag sequences (3xFlag) at the N-terminus was excised from p3xFlagCMV7.1-mPU.1 and re-ligated into the *Bgl*II/*Sal*I-digested pMXs-IG plasmid[12] to generate pMXs-IG-3xFlag-mPU.1. Plasmids pMXs-IG (mock vector) and pMXs-IG-3xFlag-mPU.1 were transiently introduced into Plat-E packaging cells to obtain infectious viruses as described previously.[12] BM-derived cells were infected with retrovirus on day −2, and GFP-positive transfectants were sorted on day 0 by using JSAN (Bay Bioscience, Hyogo, Japan).

Statistical analysis

Statistical analysis was performed using a two-tailed Student's *t*-test with p values <0.05 considered significant.

Results and discussion

Effect of PU.1 siRNA on the NFATc1 mRNA level in osteoclast

PU.1 siRNA that was previously used for specific knockdown of PU.1 mRNA,[10,12] was introduced into mouse BM-derived osteoclasts. Although the PU.1 mRNA knockdown level was moderate in comparison with that obtained in our previous studies using BM-dendritic cells (DCs),[10,12] the level of PU.1 mRNA in osteoclasts was significantly reduced by introduction of PU.1 siRNA either by

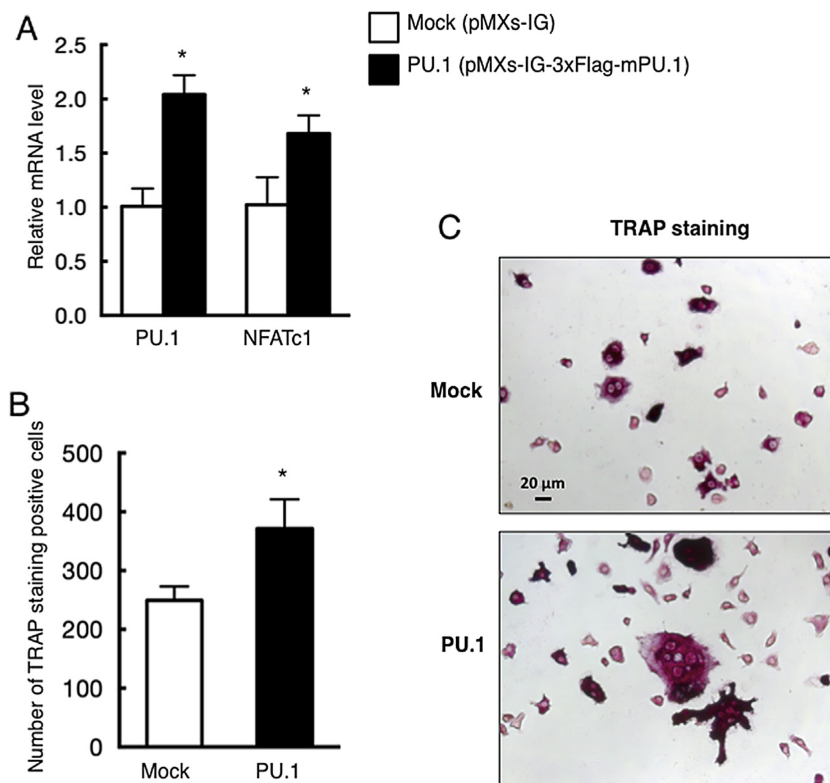

Fig. 5. Effect of enforced expression of PU.1 using a retroviral vector on the mRNA expression of NFATc1 and on TRAP activity. BM-derived cells were infected with retrovirus on day −2, and GFP-positive transfectants were sorted on day 0. Cells were harvested on day +6 for analysis of mRNA levels (A) or for TRAP staining (B, C). *$p < 0.05$.

the lipofectamine method (Fig. 1A) or by the electroporation method (Fig. 1B). Under these experimental conditions, we found that the NFATc1 mRNA level in PU.1 siRNA-introduced cells was lower than that of cells with control siRNA, indicating that the amount of PU.1 affects the NFATc1 transcriptional level.

Effect of PU.1 siRNA on NFATc1 promoter activity

The reduction in BM-derived osteoclast NFATc1 mRNA level resulting from the introduction of PU.1 siRNA (Fig. 1) suggested that PU.1 is involved in the transcription of the *NFATc1* gene. To evaluate the effects of PU.1 on NFATc1 promoter activity, we performed a reporter assay using a luciferase-linked NFATc1 promoter transfected into the RAW264.7 cell line, which is a murine monocytic cell line that was previously shown to be useful for evaluation of PU.1-mediated gene expression.[10] Luciferase assays showed that the NFATc1 promoter exhibited significant transcriptional activity when transfected into RAW267.4 cells, as shown in Fig. 2. When PU.1 siRNA was introduced into RAW267.4 cells with the reporter plasmid, the luciferase activity driven by the NFATc1 promoter decreased, whereas luciferase activity of the transfected control pGL4-Basic plasmid was not reduced by transfection of PU.1 siRNA. These results suggest that the PU.1 expression level is positively associated with NFATc1 promoter activity.

PU.1 binds to the NFATc1 promoter in osteoclasts

Knockdown of PU.1 reduced NFATc1 mRNA levels and down-regulated NFATc1 promoter activity. To determine whether PU.1 directly associates with the NFATc1 promoter region in chromosomes, a ChIP assay was performed using the BM-derived osteoclasts. As shown in Fig. 3, a markedly higher amount of chromosomal DNA containing the NFATc1 promoter was immunoprecipitated with the anti-PU.1 Ab compared with control IgG. This result indicates that PU.1 binds to the NFATc1 promoter region in osteoclasts.

PU.1 knockdown by siRNA suppresses the generation of osteoclasts

We then determined the effect of PU.1 knockdown using siRNA on the expression of osteoclast-specific genes, which we assayed using quantitative real-time PCR. A decrease in NFATc1 mRNA level was observed when the PU.1 mRNA level was reduced to approximately 30% of that of control cells on day 8. Under this experimental condition, the mRNA levels of osteoclast-specific genes, including Ctsk, Oscar, Acp5, and Itg3 were significantly decreased compared to control siRNA-transfected cells (Fig. 4A). The number of TRAP positively stained cells was also reduced by PU.1 siRNA-transfection (Fig. 4B). These results indicate that PU.1 knockdown by siRNA suppresses osteoclastogenesis.

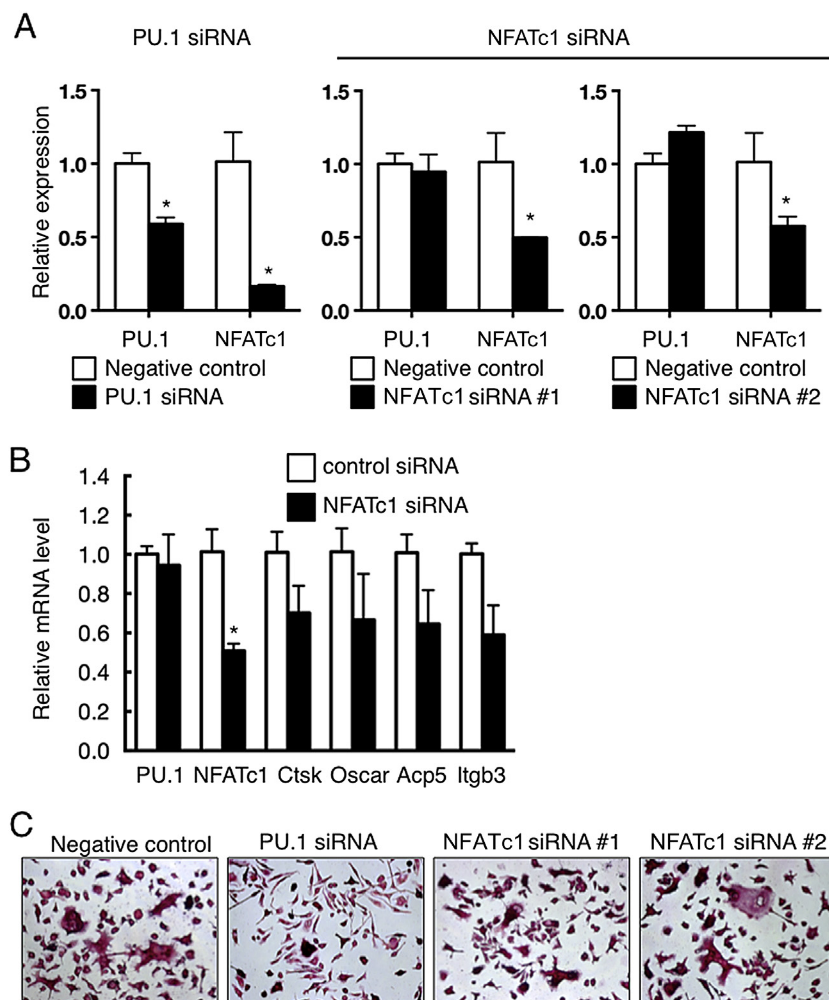

Fig. 6. Effect of NFATc1 siRNA on osteoclast-specific gene expression and function. NFATc1 siRNA or control siRNA was introduced into BM-derived cells on day 0 by electroporation and cells were harvested on day +6. A. PU.1 mRNA levels in NFATc1 siRNA-introduced cells. PU.1 siRNA-introduced cells prepared in the same experiment are shown to comparison. *$p < 0.05$. B. The mRNA expression level of osteoclast-specific genes in NFATc1 siRNA-introduced cells. *$p < 0.05$. C. TRAP staining of NFATc1 siRNA-introduced cells.

Enforced expression of PU.1 accelerates the development of osteoclasts

To confirm the involvement of PU.1 in the expression of NFATc1, BM-derived cells were transfected with a retroviral vector carrying mouse PU.1 cDNA. The PU.1 mRNA level in PU.1 retrovirus-transfected cells was approximately 2-fold of that in mock transfectants (Fig. 5A). Under this experimental condition of enforced expression of PU.1, the NFATc1 mRNA level was significantly upregulated (Fig. 5A). TRAP staining showed that retroviral overexpression of PU.1 increased the number of TRAP-positive cells (Fig. 5B, C). These results suggest that enforced expression of PU.1 induces the expression of NFATc1 and subsequently accelerates the development of osteoclasts.

NFATc1 knockdown does not affect the expression of PU.1

Although the role of PU.1 in NFATc1 expression was studied in the present study, it was unknown whether NFATc1 functions upstream of PU.1. Therefore, to evaluate the effect of NFATc1 knockdown on the expression of PU.1 and osteoclastogenesis, BM-derived cells were transfected with NFATc1 siRNA. Two different siRNAs for NFATc1 were used to exclude the possibility of any off-target effects of the siRNAs, Both siRNAs reduced the amount of NFATc1 mRNA and the reduction was comparable to the reduction in the amount of PU.1 mRNA in PU.1 siRNA-introduced cells (Fig. 6A). Under this experimental condition, the PU.1 mRNA level was not affected by the knockdown of NFATc1 (Fig. 6A), but the expression level of mRNAs for some osteoclast-specific genes and the number of TRAP-positive cells were reduced by NFATc1 siRNA transfection (Fig. 6B, C). These results indicate that PU.1 expression is not under the control of NFATc1 in osteoclasts, whereas, in contrast, PU.1 plays a role as a positive regulator of NFATc1.

PU.1 is known to directly transactivate osteoclast-specific genes.[1–3,13–15] In the present study, we showed the possibility that PU.1 knockdown suppresses osteoclast-specific genes indirectly through the repression of NFATc1 expression, in addition to a direct repressive effect (shown as a schematic drawing in Fig. 7). We have previously reported that PU.1 regulates the expression of MHC class II,[10] CD80 and CD86,[12] and TNF-α[16] in DCs, and the expression of FcεRI in mast cells.[17,18] The present study indicates that, in addition to its effects on DCs and mast cells, PU.1-targeted knockdown suppresses gene expression and function of osteoclasts.

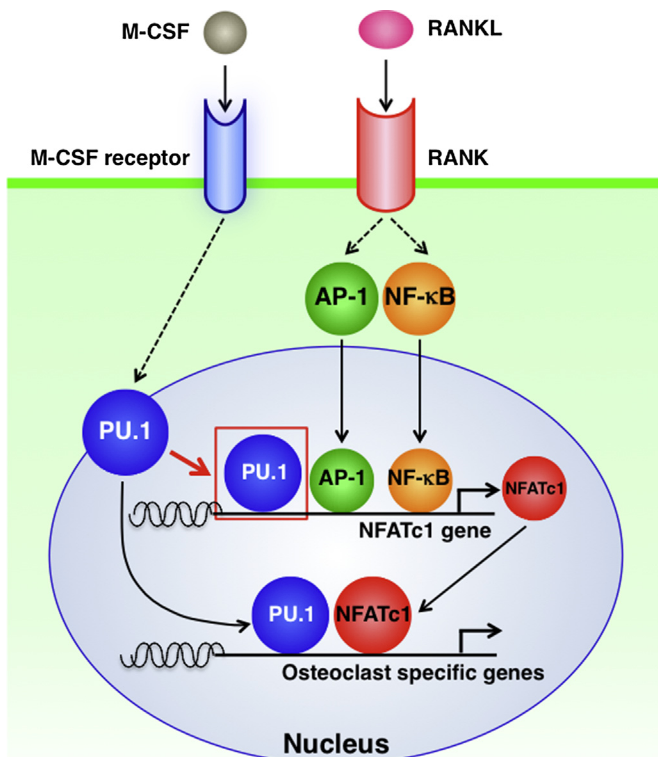

Fig. 7. Schematic drawing of the role of PU.1 in the regulation of osteoclast-specific genes. In the present study, we demonstrated that PU.1 is involved in the expression of osteoclast-specific genes as a transcriptional activator of NFATc1 (shown with the red arrow and box) in addition to the well-known role of PU.1 in the transactivation of osteoclast-specific genes, including Ctsk, Acp5, and Itgb3, in cooperation manner with NFATc1.

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Conflict of interest

FK is an employee of Bay Bioscience Corporation. The rest of the authors have no conflict of interest.

Authors' contributions

KI, TY, NN, KK, RM, MH, FK, and KM performed experiments and analyzed data; NT, KO, HO, and YT designed research; CN designed research and wrote the paper.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.alit.2015.01.006.

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