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ORIGINAL ARTICLE

Novel method for gain-of-function analyses in primary osteoclasts using a non-viral gene delivery system

Keizo Nishikawa[1,2,3] · **Masaru Ishii**[2,3]

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Abstract

Introduction Overexpression studies have been commonly used to yield significant advances in cell biology. In vitro osteoclast culturing involves the differentiation of bone marrow-derived monocyte macrophage precursors (BMMs) in medium supplemented with macrophage colony-stimulating factor and receptor activator of nuclear factor-kB ligand (RANKL) into mature osteoclasts. Retroviral vectors are the gold standards for efficient gene delivery into BMMs. While this strategy is effective in BMMs that are in the early stages of differentiation, it is ineffective in RANKL-treated BMMs such as mono- and multinucleated osteoclasts. This study attempted to enhance gene delivery into differentiated BMMs using liposome-mediated RNA transfection.

Material and methods BMMs were transfected with an EYFP overexpression plasmid or EYFP RNA by lipofection, or transduced with a retroviral vector expressing EYFP. EYFP expression was assessed by flow cytometry.

Results We performed overexpression analyses using enhanced yellow fluorescent protein (EYFP). Although EYFP expression was observed 24 h after infection of BMMs with a recombinant retrovirus containing EYFP, expression of EYFP was observed within 3 h of transfection with EYFP RNA. Moreover, the efficiency of EYFP RNA for gene delivery into BMMs was comparable to that of retroviral transduction of EYFP. In contrast, while very few BMMs stimulated by RANKL for two days expressed EYFP after retroviral infection, more than half of the cells expressed EYFP after transfection with EYFP RNA.

Conclusion RNA-mediated gene delivery is quick and easy method for performing gain-of-function analyses in primary osteoclast precursors and mature osteoclasts.

keywords Osteoclast · Primary cell culture · Retrovirus · RNA transfection

Introduction

Bone is a type of connective tissue comprising organic proteins, such as collagen, and inorganic mineral hydroxyapatite. It undergoes constant remodeling by osteoclasts and osteoblasts during our adult life [1–3]. Osteoclasts are multinucleated cells of the monocyte/macrophage lineage that degrade bone matrix. Osteoclastogenesis is tightly regulated by the binding of RANKL and macrophage colony-stimulating factor (M-CSF) to their respective receptors, RANK and c-Fms, on osteoclast precursors. RANKL is a cytokine that is crucial for cell cycle progression and osteoclast differentiation [4–6]; while, M-CSF is important for the survival and motility of osteoclast lineage cells and expression of RANK [7–9]. Signaling involving RANK and c-Fms has been extensively studied using gain- and loss-of-function approaches in cultured osteoclasts. Gain-of-function experiments have been used to identify modulators of RANK and c-Fms signaling [10–13], genes that promote survival [14–17], and transcriptional programs involved in osteoclast maturation [18–21].

✉ Keizo Nishikawa
kenishik@mail.doshisha.ac.jp

[1] Laboratory of Cell Biology and Metabolic Biochemistry, Department of Medical Life Systems, Graduate School of Life and Medical Sciences, Doshisha University, Tatara Miyakodani 1-3, Kyotanabe, Kyoto 610-0394, Japan

[2] Department of Immunology and Cell Biology, Graduate School of Medicine/Frontier Biosciences, Osaka University, Yamada-oka 2-2, Suita, Osaka 565-0871, Japan

[3] WPI-Immunology Frontier Research Center, Osaka University, Yamada-oka 2-2, Suita, Osaka 565-0871, Japan

Retrovirus-mediated gene transduction in osteoclasts cultured in vitro is the gold standard for gain-of-function analyses. However, as retroviruses are incapable of infecting non-dividing cells, this strategy is only effective in the early differentiation stages of proliferating osteoclasts, such as bone marrow-derived monocyte macrophage precursors (BMMs). Therefore, retrovirus-mediated gene transduction is not recommended for cells in the late stage of differentiation such as mono- and multinucleated osteoclasts. Alternatively, adenovirus-mediated gene delivery is a useful approach for introducing genes into RANKL-stimulated BMMs. However, the process of generating recombinant adenoviruses, including construction of an adenovirus plasmid containing the gene of interest, recombination of the plasmid with the adenoviral genome, and packaging and purification of recombinant adenovirus at high titers, is laborious and time consuming. Furthermore, the size of the transgene is substantially limited due to the small packaging capacity of the viral particle.

This study aims to tackle this problem and provides a quick and easy method for gain-of-function analyses in primary osteoclast precursors and mature osteoclasts using RNA-mediated gene introduction. Our findings are predicted to substantially help future research in bone cell biology based on gain-of-function analyses.

Materials and methods

Cell culture

In vitro osteoclast differentiation was described previously [20, 22]. Briefly, for in vitro differentiation, bone marrow-derived cells from C57BL6/J mice were cultured with 10 ng/mL M-CSF (Miltenyi Biotec) for two days and used as osteoclast precursor cells and BMMs. Subsequently, cells were further cultured with 50 ng/mL RANKL (Peprotech) in the presence of 10 ng/mL M-CSF for three days. Animal study was approved by the Institutional Animal Care and Use Committee of both Doshisha University and Osaka University.

Gene transfer of EYFP

pcDNA3-EYFP and its derivatives were constructed by inserting DNA fragments encoding EYFP, 4-amino acid sequence (Pro-Glu-Ser-Thr, PEST) [23], a destabilizing domain derived from FKBP12 protein [24], and DNA fragments of adenylate-uridylate (AU)-rich elements (ARE) [25] into pcDNA3 vector. For RNA transfection experiment, synthetic capped RNA was made with the mMESSAGE mMACHINE T7 ULTRA Transcription kit (Thermo) using linearized DNA of the pcDNA3-EYFP derivatives and then purified by RNeasy Mini kit (Qiagen). RNA transfections were performed with the Lipofectamine MessengerMAX (Thermo) according to the manufacturer's instructions. Three hours after transfection with RNA, BMMs were stimulated with 50 ng/mL RANKL. For the analysis of *EYFP* derivatives containing AU-rich elements and PEST sequences, BMMs were transfected with the *EYFP*-derivative RNA by lipofection for 3 h and then cultured with 50 ng/mL RANKL in the presence of 10 ng/mL M-CSF. Three and 48 h after RANKL stimulation, flow cytometry analysis was performed. For the analysis of the *EYFP* derivative containing the DD domain, BMMs were transfected with *EYFP*-derivative RNA by lipofection for 3 h and cultured with RANKL and M-CSF in the presence of 0.5 μM Shield-1 for one day. Then, cells were further cultured in the presence and absence of Shield-1 for one day and subjected to flow cytometry analysis.

For retroviral gene transfer experiment, the retroviral vector pMX-EYFP was constructed by inserting DNA fragments encoding EYFP into the pMX vector. Retroviral packaging was performed by transfecting the plasmids into Plat-E cells using FuGENE6 as described previously [26]. Ten hours after transduction with retroviruses, BMMs were stimulated with RANKL.

For DNA transfection experiment, pcDNA3-EYFP derivatives were transfected using FuGENE HD (Promega) according to the manufacturer's instructions. Twenty-four hours after transfection with DNA vectors, BMMs were stimulated with 50 ng/mL RANKL.

Flow cytometry analysis

The analysis was described previously [21]. Briefly, single-cell suspensions were incubated with anti-CD16/CD32 for ten minutes, and then stained with Phycoerythrin-conjugated anti-CD11b (M1/70; eBioscience) in flow cytometry buffer (1 × phosphate-buffered saline [PBS], 4% heat-inactivated fetal calf serum, and 2 mM EDTA) for fifteen minutes. Stained cells were analyzed on a FACSCanto II Flow Cytometer (BD Biosciences). FACS data were statistically analyzed with FlowJo software (TreeStar Inc.).

Quantitative RT-PCR analysis

Total RNA and cDNA were prepared using the RNeasy Mini Kit (Qiagen) and Superscript III reverse transcriptase (Invitrogen), respectively, according to the manufacturers' instructions. Real-time PCR was performed with a Thermal Cycler Dice Real Time System (TaKaRa Bio) using SYBR Premix EX Taq (TaKaRa Bio). The primer sequences were described previously [21].

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using the unpaired two-tailed Student's *t* test for comparisons between two groups (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant).

Results

To compare the efficacy of liposome and retrovirus-mediated gene delivery into osteoclast precursors, we overexpressed EYFP as our test gene of interest. When bone marrow cells are cultured with M-CSF for two days to generate BMMs that are stimulated by RANKL (with M-CSF) for three days, cells differentiate into multinucleated osteoclast-like cells (OCLs) positive for the osteoclast marker tartrate-resistant acid phosphatase capable of bone resorption [21]. Prior to treatment with RANKL, BMMs were transfected with EYFP-overexpressing plasmid or capped *EYFP* RNA by lipofection, or transduced with a retroviral vector expressing EYFP (Fig. 1A). Both *EYFP* RNA transfection and retroviral transduction yielded a higher efficiency of EYFP expression as compared to plasmid transfection (Fig. 1b). We detected fluorescence in *EYFP* RNA-transfected BMMs at 3 h and 8 h following lipofection. However, EYFP expression was barely detectable in retrovirus-transduced or plasmid-transfected BMMs. One day after gene delivery, the EYFP fluorescence was observed in BMMs transfected with *EYFP* RNA and transduced with the retroviral vector. Gene expression profiling revealed that *EYFP* RNA reached a maximum level at three hours post-transfection of *EYFP* RNA and was barely detectable by twenty-four hours post-transfection (Fig. 2a). In contrast, *EYFP* mRNA levels increased in retrovirus-transduced cells 24 h after infection. Two days after RANKL stimulation, flow cytometry showed that 69.5 ± 1.7% and 62.4 ± 3.8% of CD11b-positive cells transfected with *EYFP* RNA and retrovirus-transduced cells were positive for EYFP protein expression, respectively (Fig. 2b).

When BMMs transfected with *EYFP* RNA or transduced with retroviral vector were further treated with M-CSF and RANKL, the fluorescent signal of EYFP was observed in multinucleated cells three days after gene delivery (Fig. 2c). However, the fluorescence intensity of EYFP was reduced in BMMs transfected with *EYFP* RNA, consistent the negligible detection of exogenous EYFP RNA (Fig. 2a). Together, these results indicate that the efficiency of gene delivery by *EYFP* RNA lipofection and retroviral transduction of EYFP was comparable in BMMs. Transfection of RNA is a feasible approach to overexpress exogenous proteins through different stages of osteoclast differentiation (very early to late stage of osteoclasts).

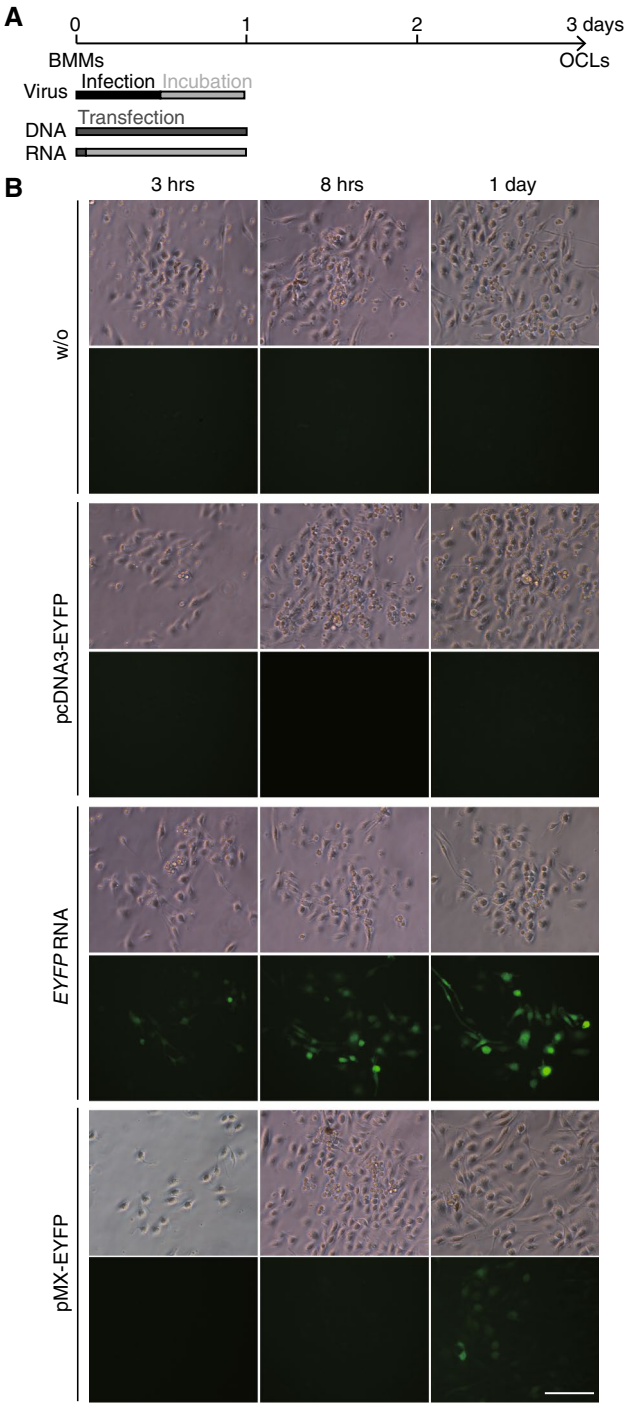

Fig. 1 Gene delivery of the exogenous protein, EYFP, into osteoclast precursors. **a** Schematic of the protocol for gene delivery into bone marrow-derived monocyte macrophage precursors (BMMs). For retroviral transduction, BMMs were transduced with a retroviral vector expressing EYFP for 10 h. For lipofection, BMMs were incubated with lipid–RNA or –DNA complexes for 3 h or 24 h, respectively. **b** Representative fluorescence images of EYFP in BMMs transfected with the EYFP-overexpressing plasmid or *EYFP* RNA by lipofection, or transduced with a retroviral vector expressing EYFP. Scale bar 100 μm

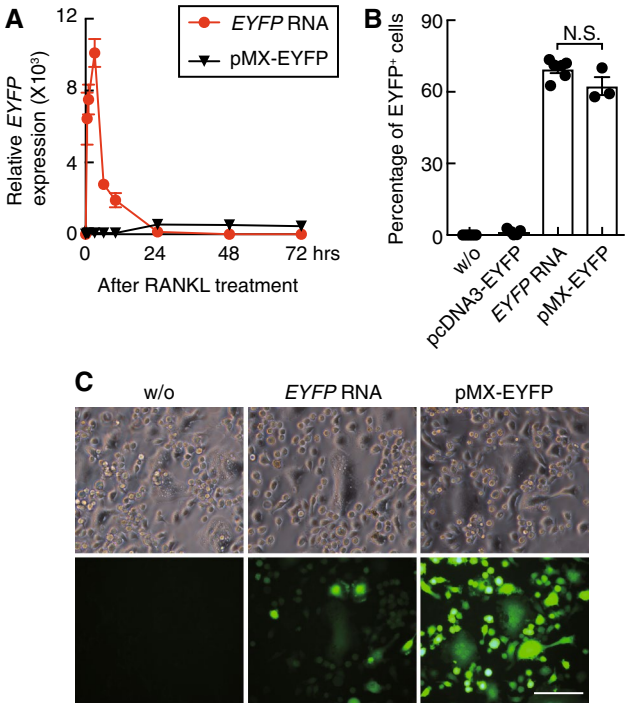

Fig. 2 Expression of EYFP in osteoclast precursors and mature osteoclasts. **a** Gene expression profiles of *EYFP* in BMMs transfected with EYFP. BMMs were transfected with the *EYFP* RNA by lipofection for 3 h or transduced with a retroviral vector expressing EYFP for 10 h, and then cultured with RANKL in the presence of M-CSF. Gene expression was analyzed at each time point after RANKL stimulation. Percentage of EYFP-expressing cells (gated using CD11b+). BMMs were transfected with an EYFP overexpression plasmid for 24 h or *EYFP* RNA for 3 h by lipofection, or transduced with a retroviral vector expressing EYFP for 10 h, and then cultured with RANKL in the presence of M-CSF. Two days after RANKL stimulation, flow cytometry analysis was performed. **c** Representative fluorescence images of EYFP in BMMs subjected to gene introduction. BMMs were transfected with *EYFP* RNA for 3 h by lipofection, or transduced with a retroviral vector expressing EYFP for 10 h, and then cultured with RANKL in the presence of M-CSF for three days. Scale bar 100 μm

Next, we compared the efficacy of gene delivery by liposome transfection and virus transduction in fully differentiated osteoclasts. Osteoclast lineage-committed cells were induced by treating BMMs with M-CSF and RANKL. In vitro osteoclast differentiation results in the formation of multinucleated OCLs that exhibit characteristics of mature osteoclasts three days after RANKL stimulation [20]. We found that transfection with the EYFP overexpression plasmid or capped *EYFP* RNA by lipofection, or transduction with a retroviral vector expressing EYFP, of BMMs prior to and one day following RANKL-stimulated produced similar results (data not shown). Fluorescence from EYFP was observed within 3 h of transfection with *EYFP* RNA but not with the EYFP overexpression plasmid. In contrast, fluorescence from EYFP was observed 24 h after incubation with

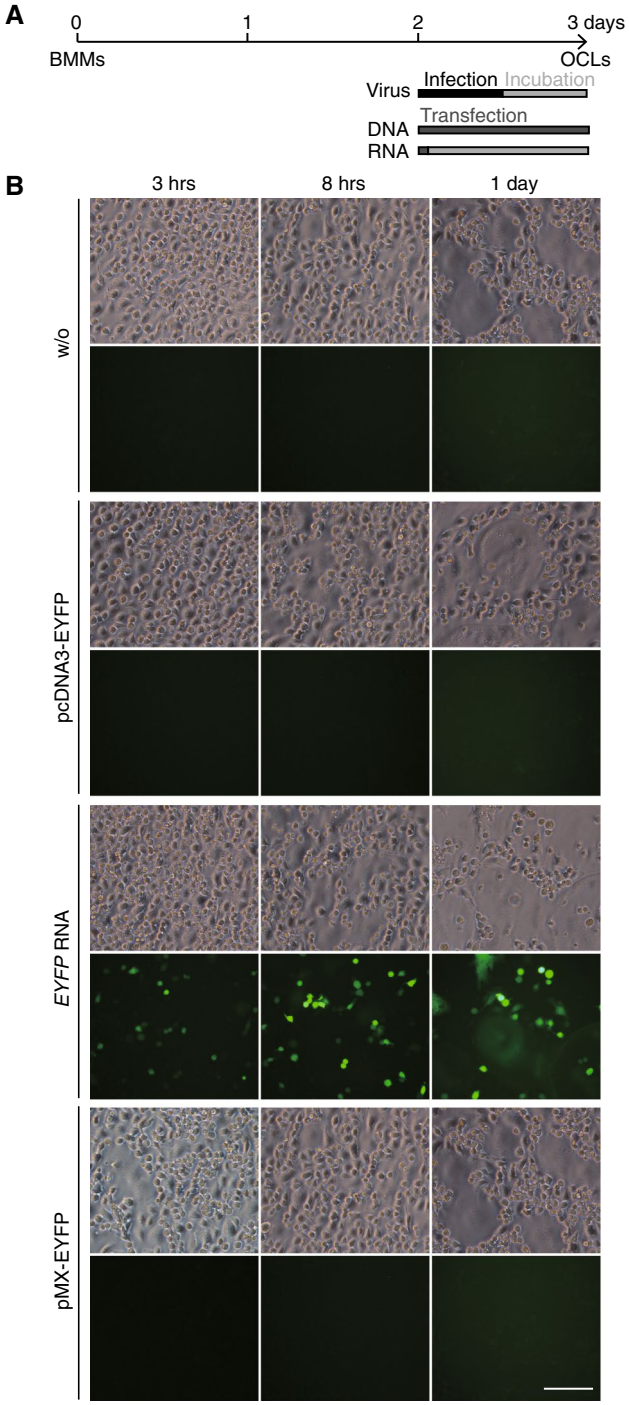

Fig. 3 Gene delivery of EYFP in fully differentiated osteoclasts. **a** Schematic of the protocol for gene delivery into BMMs two days after treatment with RANKL. For retroviral transduction, RANKL-treated BMMs were transduced with the retroviral vector expressing EYFP for 10 h. For lipofection, RANKL-treated BMMs were incubated with lipid–RNA or –DNA complexes for 3 h or 24 h, respectively. **b** Representative images for the fluorescence in fully differentiated osteoclasts transfected with the EYFP overexpression plasmid or *EYFP* RNA by lipofection, or transduced with the retroviral vector expressing EYFP. Scale bar 100 μm

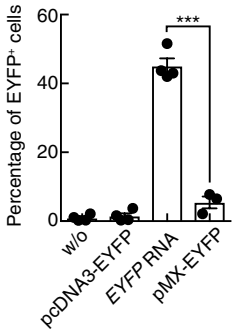

Fig. 4 Expression of EYFP in mature osteoclasts. RANKL-treated BMMs for two days were transfected with an EYFP overexpression plasmid for 24 h or *EYFP* RNA for 3 h by lipofection, or transduced with a retroviral vector expressing EYFP for 10 h, and further cultured with RANKL in the presence of M-CSF. One day after gene introduction, the percentage of EYFP-expressing cells (gated using CD11+) was analyzed by flow cytometry

the *EYFP*-containing retrovirus. There was a significant difference in the level of fluorescence in liposome-transfected or retrovirus-transduced BMMs stimulated with RANKL for two days (Fig. 3a). Very few cells expressed EYFP 24 h after infection with the recombinant retrovirus (Fig. 3b), while approximately 50% of cells expressed EYFP after transfection with *EYFP* RNA (Fig. 4). Interestingly, fluorescence from EYFP was detected in multinucleated OCLs as well as mononucleated cells (Fig. 3b). These results suggest that RNA-based gene delivery is effective in osteoclast precursors and mature osteoclasts.

Based on these findings, we aimed to develop a method to transiently overexpress exogenous proteins selectively during the early stage of osteoclast differentiation. We used the following approach: destabilization of exogenous protein at the RNA and protein level during late stage osteoclast differentiation. AREs are a major *cis*-acting binding element in the untranslated region of labile RNAs [25]. The PEST tag comprises a peptide sequence that is rich in proline, glutamic acid, serine, and threonine, and reduces the half-life of PEST-tagged proteins [23]. BMMs transfected with *EYFP* RNA harboring either AREs or PEST tags were treated with M-CSF and RANKL showed no reduction in EYFP fluorescence throughout osteoclast differentiation (data not shown). Fluorescence from EYFP containing both the AREs and PEST tags decreased to a slight yet significant extent during the late stages of osteoclast differentiation (Fig. 5a). Next, we used the Shield-1 ligand to rapidly increase the stability of EYFP [24]. This technique involves the fusion of the protein of interest to a destabilizing domain (DD) derived from FKBP12 protein. Addition of the membrane permeable small molecule, Shield-1, that binds to the DD shields the protein from degradation. Removal of Shield-1 leads to rapid degradation of the DD domain along with any fused protein. BMMs were transfected with *EYFP* RNA carrying the DD domain and treated with M-CSF and RANKL in the presence of Shield-1. One day post-transfection, although

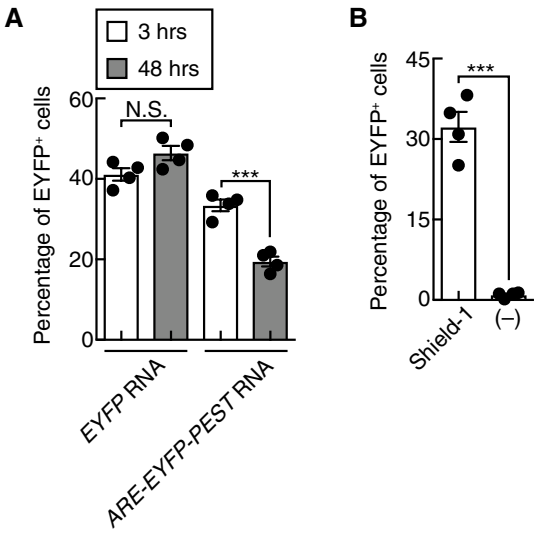

Fig. 5 Expression of EYFP derivatives in osteoclast precursors. **a** Percentage of EYFP-expressing cells (gated using CD11+) after transfection with *EYFP* derivative containing AU-rich elements and PEST sequences. BMMs were transfected with the *EYFP* derivatives RNA by lipofection for 3 h and then cultured with RANKL in the presence of M-CSF. Three and 48 h after RANKL stimulation, flow cytometry analysis was performed. **b** Percentage of EYFP-expressing cells (gated using CD11s+) after transfection with *EYFP* derivative containing DD domain. BMMs were transfected with the *EYFP* derivatives RNA by lipofection for 3 h and cultured with RANKL and M-CSF in the presence of Shield-1 (0.5 μM) for one day. Then, cells were further cultured in the presence and absence of Shield-1 for one day and were subjected to flow cytometry analysis

RANKL-treated BMMs exhibited fluorescence from EYFP, removal of Shield-1 completely and rapidly reduced the fluorescence from EYFP (Fig. 5b). Taken together, our method of RNA-mediated gene delivery overexpresses exogenous proteins based on the various stages differentiation in primary osteoclasts.

Discussion

To the best of our knowledge, this is the first report on a quick and easy method for overexpressing exogenous proteins in primary osteoclast precursors and mature osteoclasts. Overexpression experiments constitute a qualitative method to identify novel proteins and investigate their function. Overexpression of NFATc1 induces osteoclastogenesis even in the absence of RANKL, suggesting that NFATc1 is a master regulator of osteoclast differentiation [18]. Thus, overexpression experiments have become an indispensable technique in molecular biology research.

In primary cell cultures, including the in vitro osteoclast differentiation assay, virus-mediated gene transduction is the gold standard for overexpression experiments; however, this technique is associated with drawbacks, including limited size of the gene of interest and physiological stage of cells undergoing gene transduction. RNA-mediated gene delivery helps to overcome these drawbacks and provides a powerful approach to study osteoclastogenesis. Despite the efficacy of gene delivery by RNA-mediated method, however, adenoviral transduction may still be advantageous in some studies as it has been used extensively for gain-of-function analyses and allows for efficient gene delivery into both osteoclast precursors and mature osteoclasts [27–29].

Bacterial CRISPR–Cas9 has been commonly used for genome editing and has emerged as a multifunctional platform for sequence-specific regulation of gene activation and repression, modification of CpG methylation, and visualization of genomic loci [30, 31]. However, since Cas9 is large protein, the virus has a low efficiency of encapsulating Cas9. Moreover, purifying recombinant viruses in high titer is laborious and time consuming. The major advantages our method of RNA-mediated gene delivery include speed and simplicity (regardless of size limitation). Therefore, combining CRISPR with our technique can be applied to cultures of primary osteoclasts.

We used techniques in destabilizing RNA and protein of exogenous genes to successfully overexpress exogenous proteins specifically in differentiating primary osteoclasts. Tools in optogenetics have been developed to enable the optical enhancement of protein stability [32] or degradation [33]. Therefore, protein levels can be rapidly fine-tuned by utilizing optogenetic tools.

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Compliance with ethical standards

Conflict of interest All authors state that they have no conflicts of interest.

Ethical approval Animal study was approved by the Institutional Animal Care and Use Committee of both Doshisha University and Osaka University.

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