

RETRACTED ARTICLE: Efficient miRNA Inhibitor with GO-PEI Nanosheets for Osteosarcoma Suppression by Targeting PTEN

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Background: Gene therapy is considered a novel way to treat osteosarcoma, and microRNAs are potential therapeutic targets for osteosarcoma. miR-214 has been found to promote osteosarcoma aggression and metastasis. Graphene oxide (GO) is widely used for gene delivery for the distinct physicochemical properties and minimal cytotoxicity.

Methods: Polyethyleneimine (PEI)-functionalized GO complex was well-prepared and loaded with miR-214 inhibitor at different concentration. The load efficacy was tested by gel retardation assay and the cy3-labeled fluorescence of cellular uptake. The experiments of wound healing, immunofluorescence staining, Western blot, qRT-PCR and immunohistochemical staining were performed to measure the inhibitory effect of the miR-214 inhibitor systematically released from the complexes against MG63, U2OS cells and xenograft tumors.

Results: The systematic mechanistic elucidation of the efficient delivery of the miR-214 inhibitor by GO-PEI indicated that the inhibition of cellular miR-214 caused a decrease in osteosarcoma cell invasion and migration and an increase in apoptosis by targeting phosphatase and tensin homolog (PTEN). The synergistic combination of the GO-PEI-miR-214 inhibitor and CDDP chemotherapy showed significant cell death. In a xenograft mouse model, the GO-PEI-miR-214 inhibitor significantly inhibited tumor volume growth.

Conclusion: This study indicates the potential of functionalized GO-PEI as a vehicle for miRNA inhibitor delivery to treat osteosarcoma with low toxicity and miR-214 can be a good target for osteosarcoma therapy.

Keywords: osteosarcoma, GO-PEI-miR-214 inhibitor, tumor suppression

Introduction

Osteosarcoma is one of the most common types of aggressive malignant bone tumors.¹ Since the high invasiveness and rapid metastasis, the conventional therapy of osteosarcoma was subject to serious restriction. microRNAs (miRNA) have acted as important prognostic factors in patients with osteosarcoma,^{2,3} and miR-214 was proved to be linked to osteosarcoma progression and adverse prognosis.⁴⁻⁶ The depletion of miR-214 or miR-214 inhibition increased radiosensitivity or chemosensitivity in both osteosarcoma cells and mouse xenograft models.^{4,7} However, naked miRNAs or miRNA inhibitors can be rapidly degraded in culture medium or in vivo. Therefore, vectors that protect and deliver miRNAs or miRNA inhibitors into cells are imperative for miRNA therapy.

Many gene delivery tools have been developed, including liposomes, viruses, polymer particles, carbon nanomaterials, etc.^{8,9} Viral vectors are efficient gene delivery

but the biosafety concerns limit its extensive application.¹⁰ However, the transfection efficiency of nonviral vectors is low and needs to be improved.¹¹ The major obstacle is to develop nonviral-based safe and efficient gene delivery vehicles.

Graphene oxide (GO) nanosheets have been demonstrated to utilize in drug delivery for cancer therapy for their abundant oxidized functional groups and large surface area to adsorb nucleobases or aromatic compounds.^{12–14} In this study, we designed a GO-PEI complex to deliver the miRNA into cells in vitro and in vivo. Negatively charged GO is able to bind cationic polyethyleneimine (PEI) polymers to form stable GO-PEI complexes by electrostatic interactions. GO-PEI complexes were highly enriched in positive charges that could bind to negative miRNA and wrap miRNA. Therefore, it was hypothesized that the highly enriched positive charges of GO-PEI complexes allow effective loading of miRNA inhibitors and delivery inhibitors into cells for tumor suppression. To test this hypothesis, we delivered miR-214 inhibitor into two different osteosarcoma cell lines by means of GO-PEI complexes. And the progression of osteosarcoma was inhibited by GO-PEI-miR

-214-inhibitor (GP-inhibitor) complexes through PTEN/PI3K/Akt and ERK1/2 signaling pathways. A schematic diagram was shown that GP-inhibitor delivered to osteosarcoma cells for therapy in Figure 1.

Experiments and Methods

Preparation of GO-PEI

The GO-PEI complexes were obtained by GO (Aladdin, Shanghai, China) conjugated with PEI (Sigma-Aldrich, Missouri, USA) through electrostatic adsorption. Briefly, PEI solutions were mixed with diluted GO solution at a GO:PEI weight ratio of 1:3. A 25-kDa PEI solution (1 mg/mL) was added to a GO solution (approximately 1 mg/mL) in 10 min. The mixture was ultrasonicated for 15 min and stirred overnight. The mixed solution was washed with ddH₂O and centrifuged at 3000 rpm, 5 min, 4°C and filtered. The size distributions of GO and GO-PEI were measured by a DLS spectrophotometer (Tosaka Electronics, Japan). The FTIR spectrum was used to test the presence of functional groups on the surface of GO, PEI, and GO-PEI complexes. The spectrum was measured from 4000 to 400 cm⁻¹ using an FTIR instrument (Vertex 70, Bruker, Germany) at room

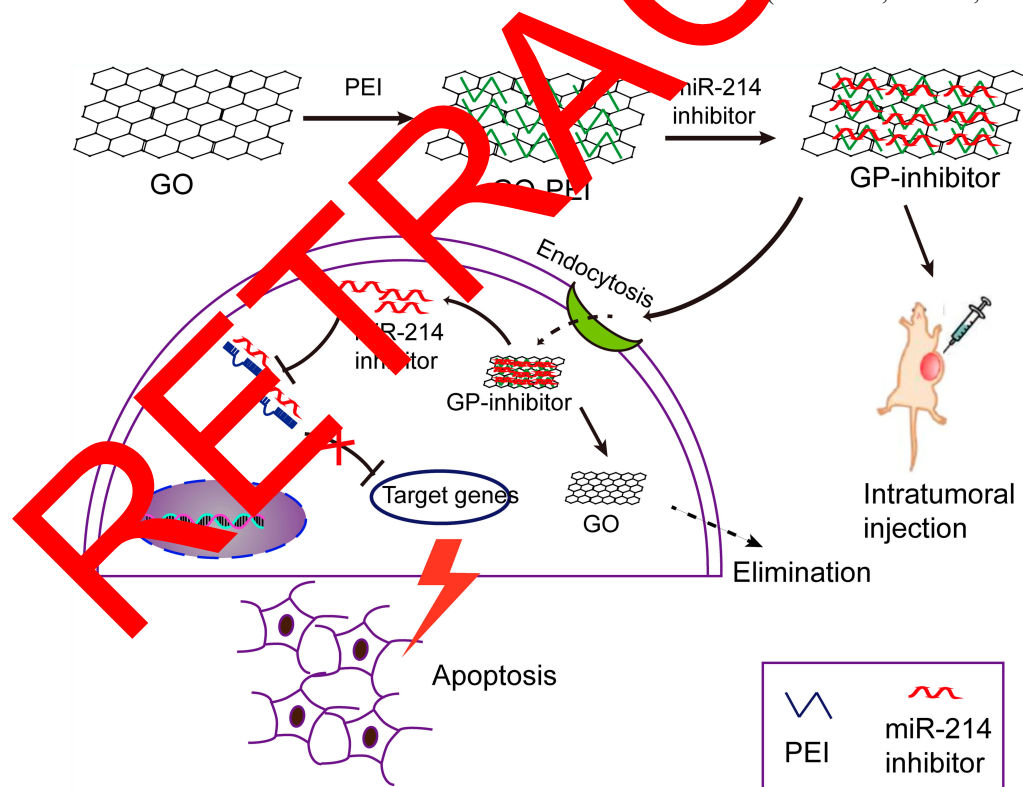


Figure 1 A schematic diagram depicting the efficiency of miR-214 inhibitor delivery into cells by means of graphene oxide (GO)-polyethyleneimine (PEI). GO was conjugated with PEI polymers to form positively charged GO-PEI complexes. Negatively charged miR-214 inhibitor was wrapped into the GO-PEI complexes by electrostatic interactions. GP-inhibitor complexes were delivered into the cytoplasm through endocytosis and inhibited the expression of miR-214 in osteosarcoma cells. Due to miR-214 inhibited by GP-inhibitor, the expression of PTEN could be increased and demonstrated a therapeutic effect in vitro. The GP-inhibitor also showed good therapeutic effect on the subcutaneous xenograft tumor mouse model through intratumoral injection.

temperature. The samples suspended in deionized water were analyzed the surface charge using a Zeta-sizer (Malvern Nano ZS, Malvern, UK). GO and GO-PEI were also examined by TEM (HT7700, Hitachi, Japan) analysis. The ultraviolet-visible (UV-vis) absorption spectrum (190 to 800 nm) for GO-PEI and GO-PEI-inhibitor (10 $\mu\text{g/mL}$) samples was obtained by a UV-vis spectrophotometer (Shimadzu, Japan). To determine the gel retardation assay, the GO-PEI-miRNA inhibitor solution (9 μL) was added with 3 μL of 4 $\times$ loading buffer, loaded on a 1% (w/v) agarose gel and then electrophoresed at 100 V for 25 min.

Cell Viability Assay

Human U2OS and MG-63 cell lines (Genesee, Guangzhou, China) were used in this study. The CCK-8 assay was applied to measure the cytotoxicity of GO and GO-PEI complexes. Briefly, U2OS and MG-63 cells were cultured with GO-PEI at a series of concentrations (0, 5, 10, 20, 30, 40 and 50 $\mu\text{g/mL}$) for 24 hrs. Then, the CCK-8 test solution was added to each well and incubated at 37°C for 2 hrs. The absorbance of each well was measured at 450 nm using a microplate reader (Molecular Devices, CA, USA). The experiment was repeated 3 times.

Measurement of Cellular Uptake of GO-PEI

To measure the cellular uptake of GO-PEI, GO-PEI was stained with FITC dye.¹⁵ GO-PEI and FITC-BSA solution (Bioss Inc., Beijing, China) (1 mg/mL) were mixed with a mass ratio of 1:1 at 37°C and incubated for 4 hrs, the supernatant containing any unbound FITC-BSA was discarded after centrifuged at 10,000 g. For the cellular uptake test, U2OS and MG-63 cells were seeded in 24-well plates (5×10^4 /well) and then incubated with different doses of FITC-labeled GO-PEI in serum-free medium for 24 h. GO-PEI-treated U2OS and MG-63 cells were fixed with 4% (w/v) paraformaldehyde at 4°C for 15 mins. The cell cytoskeleton was stained with rhodamine phalloidin (1:100, Invitrogen, CA, USA) for 30 mins at 4°C and then incubated with 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, USA) (1:2000) for 15 mins. The stained cells were examined under a laser scanning confocal microscope (Carl Zeiss, Inc, Jena Germany).

RNA Delivery and RNase Protection Assay of GP-Inhibitor

To analyze the miRNA inhibitor delivery efficiency of GO-PEI, U2OS and MG-63 cells were seeded in 24-well plates (5×10^4 /well) overnight before transfection. GO-PEI was

mixed with a cy3-conjugated miRNA inhibitor (N/P ratio of 30) for 1 h and then added into U2OS and MG-63 culture media (5 $\mu\text{g/mL}$) for 1, 4, 8, 16, 24, 36, 48 and 72 hrs. The miR-214 inhibitor was delivered by Lipofectamine 2000 (Life Technologies, CA, USA) taken as one of the controls according to the manufacturer's instructions. Cy3-miRNA inhibitor delivery was analyzed by fluorescence microscopy (Olympus Co., Ltd., Japan). To evaluate the stability of GP-inhibitor complexes in RNase A solution (30 $\mu\text{g/mL}$, Sigma), miR-214 inhibitor and GO-PEI were added simultaneously or GP-inhibitor complex were added into the solution. The cells were observed using a fluorescence microscope, and the fluorescence intensity was analyzed.

Invasion Assay and Wound Healing

To measure whether the GP-inhibitor inhibited the biological function of osteosarcoma cells, transwell assays and wound healing assays were performed. For the invasion assay, matrigel (BD Biosciences, CA, USA, 25 $\mu\text{g}/50 \mu\text{L}$) was added onto the upper well with 8 μm pore size polycarbonate membrane filters. DMEM culture medium (containing 10% FBS) was added into the lower layer of the transwell. MG63 and U2OS cells were added into the upper chamber with FBS free culture medium (5 $\mu\text{g/mL}$ lipo-inhibitor or GP-inhibitor). After 24 h of incubation, the cells in the upper well were fixed with 4% paraformaldehyde and stained with 0.4% crystal violet. Cells in the upper part of the filter were removed, and the number of stained migrated cells was counted under an inverted microscope (Olympus Co., Ltd., Japan). For the wound healing, the confluent MG63 cells cultured in six-well plates were wounded with a yellow tip (tip of 100 μL), and the cells were observed to migrate over a cell-free gap after treatment with lipo-inhibitor or GP inhibitor (5 $\mu\text{g/mL}$) for 0, 24 and 48 hrs. The cells were then examined under a microscope (Olympus Co., Ltd., Japan).

Immunofluorescence and Western Blotting Analysis

MG63 cells were seeded onto coverslips in 24-well plates (5×10^4 /well) and co-treated with lipo-inhibitor or GP-inhibitor for 24 h. The cells were fixed and permeabilized as described,¹⁶ blocked in 3% horse serum, and incubated with the primary antibody (1:250 dilution) overnight at 4°C. The primary antibody was specific for p-PTEN (CST, MA, USA) and cleaved caspase-3 (ab2302, Abcam, MA, USA), and the secondary antibody (1:300, Jackson ImmunoResearch, PA,

USA) was incubated for 1 h at room temperature and counterstained with DAPI. The images were visualized with a fluorescence microscope and analyzed by ImageJ. For Western blot analysis, cells were collected and lysed for protein, and the same amount of proteins was loaded onto a 12% SDS-PAGE gel (Beyotime, Nanjing, China) and transferred onto PVDF membranes. The membranes were blocked and incubated with monoclonal antibodies directed against p-PTEN, PTEN, p-Akt, Akt, p-ERK1/2, ERK1/2, cleaved caspase-3 and cleaved caspase-8 (CST, MA, USA), followed by HRP-conjugated secondary antibodies (CST, MA, USA). GAPDH (CST, MA, USA) was used as an internal control. The transferred membranes were reacted with an ECL Western blot substrate kit (Beyotime, Nanjing, China) for exposure and analyzed using a FluorChem™ M System (Protein Simple, CA, USA).

qRT-PCR Analysis

Total RNA or miRNA were isolated by RNeasy Mini or miRNeasy Mini kits (Qiagen, Valencia, CA, USA). The mRNA levels for genes were determined using SYBR Green qRT-PCR mix (Promega, Madison, USA) on a 7500 Fast Real-Time PCR System (Applied Biosystems, MA, USA). The expression of miR-214 was measured using the Bulge-Loop™ miRNA qRT-PCR kit (Ribobio, Guangzhou, China). Primers used for qRT-PCR are listed below: twist, forward, TTCTCGGTCTGGAGGATGGA; reverse, CATTTTCTCCTTCTCTGGAAACA; N-cadherin, forward, TGGGAAATGGAAACTTGATGGC; reverse, GATGCTCATCTGCTCTCATA; E-cadherin, forward, AGTCAATTCAGACTCCAGCC; reverse, TGTGGCTCCGGCGTCAAA; GAPDH, forward, GTCAGGCTGAGACGGGAAG; reverse, GAGGGGGCAAGATCATGACC.

In vivo Antitumor Efficacy

Six-week-old female BALB/c nude mice (20–22 g) were obtained from the Southern Medical University Animal Center. The animal experiments were performed with the approval of Jinan University Institutional Animal Care and Use Committee and followed the guidelines of the National Act on the Use of Experimental Animals (China). Five mice were housed per cage in a temperature and humidity-controlled room with a 12 hr light/dark cycle. All mice had free access to sterilized water and food.

MG63 cells (1×10^6 cells) suspended in 100 μ L of Matrigel were subcutaneously injected into the right flank of female BALB/c nude mice. Once the tumor volume reached approximately 100 mm³, the mice got the treatment

of PBS, miR-214 inhibitor, GO-PEI, and GP inhibitor by peritumoral injection. The dosage of miR-214 inhibitor was 1 mg/kg per mouse, and PEI-miR-214 inhibitor was 5 mg/kg per mouse. The beginning of the treatment was defined as day 0, and the formulations were given to the mice on days 0, 4, 8, and 12. The mice were weighed, and tumor size was measured with a Vernier caliper at different time points. The tumor volume was calculated as follows: tumor volume = length $\times$ width²/2. Each tumor volume was independently measured, and fold changes in volume were calculated according to the tumor volume on day 0.

Histology and Immunohistochemistry

The mice were anesthetized and sacrificed at day 20, and the xenograft tumors were isolated and fixed with 4% paraformaldehyde. For immunohistochemical staining, sections were blocked with 1% goat serum in PBS for 30 min, followed by the primary antibody (1:200 dilute) incubation at 4°C overnight. The primary antibodies were used for p-PTEN, caspase-3 and caspase-8 (CST, MA, USA) and followed by the secondary antibody incubation for 2 h at room temperature. Three samples for each group were measured by light microscopy (Olympus, Tokyo, Japan).

Statistical Analysis

The data are presented as the mean $\pm$ SD (standard deviation). Statistical comparisons between different groups or two groups were evaluated by one-way ANOVA test or *t*-test comparison. Statistical significance was determined with a P-value <0.05.

Results

Characterization of GO-PEI Complexes

It has been reported that GO needs to be modified for loading nucleic acids, and PEI is known as the gold standard of cationic polymers for gene transfection.^{17,18} GO-PEI complexes were synthesized at a GO: PEI weight ratio of 1:5. Pristine GO and GO-PEI were measured by transmission electron microscopy (TEM). It revealed that GO sheets might be multiple layers and highly agglomerated, GO-PEI slightly increased the surface area (Figure 2A). Dynamic light scattering (DLS) results showed that the size distributions of GO and GO-PEI were 158.6 ± 46.8 nm and 205.5 ± 65.9 nm, respectively (Figure 2B). The zeta potential of GO was negative (-32.06 ± 3.26 mV), and that of GO-PEI was positive (28.17 ± 5.09 mV), indicating that the negatively

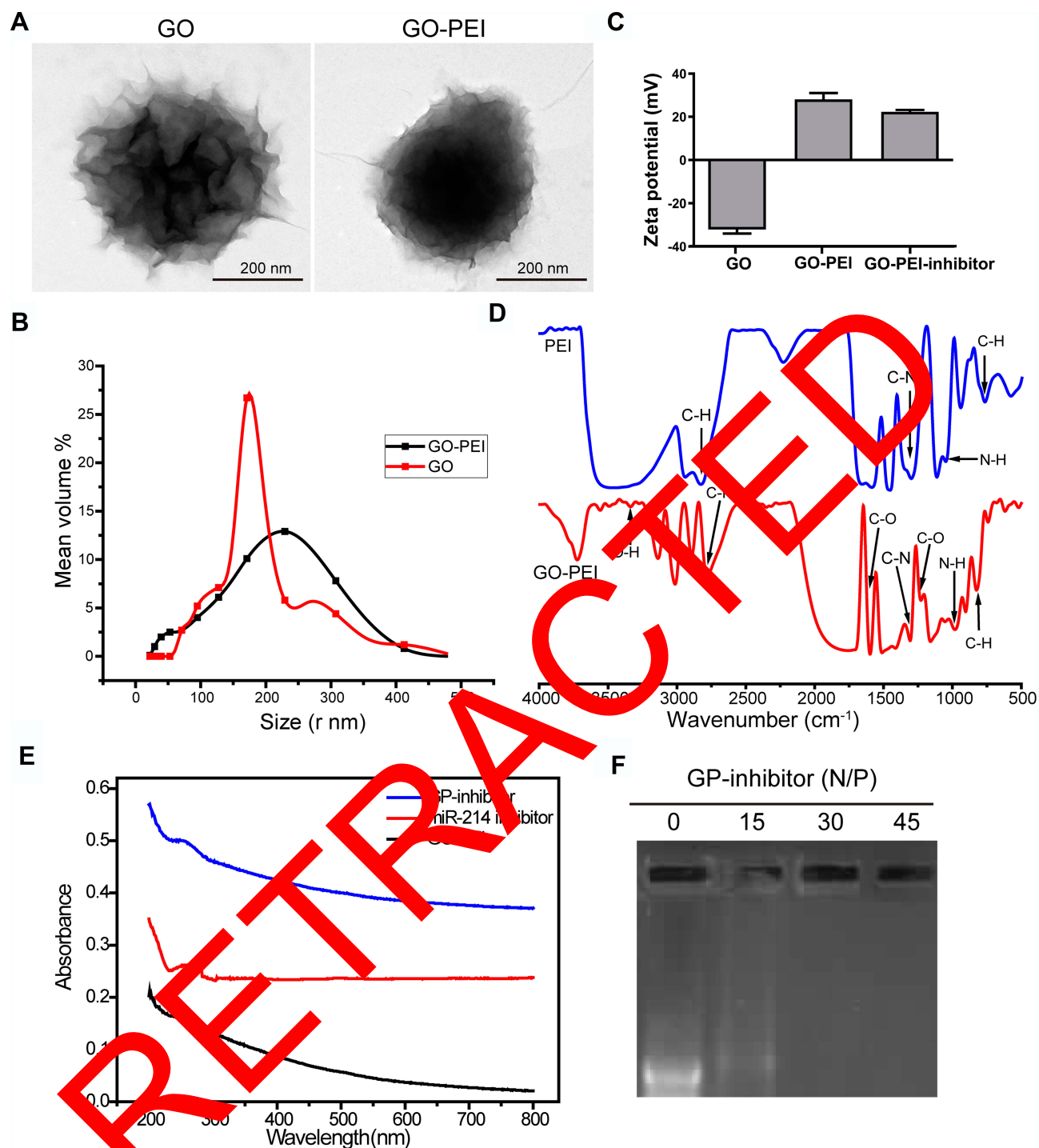


Figure 2 Characterization of GO-PEI complexes. **(A)** SEM images of GO and GO-PEI complexes. Scale bars: 200 nm. **(B)** The size distributions of GO and GO-PEI were detected by DLS analysis. **(C)** Measurement of the zeta potential of GO and GO-PEI complexes. **(D)** FTIR spectra of PEI (top) and GO-PEI complexes (bottom). **(E)** UV-vis spectra normalized by their extinction coefficients at 260 nm. Blue line for GO-PEI-miR-214 inhibitor complex, red line for free miR-214 inhibitor sense strand, and dark line for GO-PEI; experiments were carried out in phosphate buffer (pH 7.4). **(F)** A gel retardation assay of the GP-inhibitor at different N/P ratios (0, 15, 30, 45).

charged GO is able to bind cationic PEI polymers by electrostatic interactions and the conjugated PEI changed the negative charge on the surface of GO.¹² Moreover, the potential of the GO-PEI inhibitor was slightly lower than

that of GO-PEI, which meant that the negatively charged miR-214 inhibitor might be coupled with GO-PEI (Figure 2C). It is suggested that the GO-PEI complex can bond with negatively charged miRNA inhibitors to deliver

miRNA inhibitors into cells. Fourier transform infrared red (FTIR) analysis confirmed that GO-PEI and PEI showed four peaks at 665–910, 720–725, 1020–1250, and 2850–3000 cm^{-1} denoting C-H, N-H, C-N and C-H groups, respectively. GO-PEI possessed all peaks of GO and PEI, with peaks in the 1000–1320, 1665–1760, and 3200–3500 cm^{-1} ranges corresponding to the C-O, C=O, and O-H groups, respectively. These peaks indicated that PEI was successfully grafted onto GO by electrostatic interactions rather than by covalent bonding (Figure 2D). The UV-vis spectrum demonstrated an absorption peak at 280 nm for miRNA and GP-inhibitor, which meant that the miRNA inhibitor was loaded on GO-PEI (Figure 2E). To further examine the binding capacity of GO-PEI complexes to miR-inhibitor, we used agarose gel electrophoresis. GO-PEI was complexed with the miR-inhibitor at various N/P ratios (molar ratio of nitrogen of PEI to phosphate groups of miRNA). The GPM complex demonstrated significant electrophoretic retardation at the N/P ratio of 30, which meant that GO-PEI would wrap miR-inhibitor inside and prevent miR-inhibitor being degraded at the N/P ratio of 30 (Figure 2F).

Cellular Uptake of GO-PEI Analysis

To ensure that the uptake of GO-PEI complexes by cells was the first important step for GO-PEI-inhibitor delivery, TEM images were used to confirm cellular uptake. As shown in Figure 3A, GO-PEI sheets were clearly identified enclosed in a membrane in the cytosol. In addition, FITC-labeled GO-PEI was also used to track the cellular uptake and localization of GO-PEI in MG63 and U2OS cells through laser scanning confocal microscopy (LSCM). FITC-labeled GO-PEI (10 $\mu\text{g}/\text{mL}$, green fluorescence) was observed to be inside the cells or bind on the plasma membrane after 8 h of incubation, and more GO-PEI complexes were taken up into the cells as the incubation time increased. An enlarged image of GO-PEI uptake was shown in the right panel of Figure 3B. Next, the cytotoxicity of GO-PEI to MG63 and U2OS cells was measured by a cell counting kit (CCK-8) assay. The cytotoxicity of MG63 or U2OS cells had no significant difference in different GO-PEI-treated groups (5, 10, 20, and 30 $\mu\text{g}/\text{mL}$). The cells showed mild toxicity only when the concentration of GO-PEI reached 40 or 50 $\mu\text{g}/\text{mL}$. Moreover, GO-PEI-treated MG63 and U2OS cells were viable after 24 or 48 h (Figure 3C). These GO-PEI complexes showed low cytotoxicity, even the high-molecular-

weight PEI (25 kDa) was used, suggesting that GO-PEI complexes were much safer than the single PEI polymers.

GO-PEI-Inhibitor Synthesis and Transfection Evaluation

To determine whether miRNA inhibitors were successfully transfected into cells by GO-PEI complexes, a cy3-labeled miRNA inhibitor was used to study the gene delivery. MG63 cells were incubated with free miR-214 inhibitor, GO-PEI, and different N/P ratios of GO-PEI-miR-214 inhibitor. After 24 h of incubation, the cells were detected by fluorescence microscopy. The transfection efficiency was highest while the N/P ratio (GO: PEI: inhibitor) was 30, and the relative fluorescence of cy3-labeled miRNA inhibitor in cells was weaker when the N/P ratio was below 30 (Figure 4A). The GO-PEI complexes could release the inhibitors slowly in the cells and the fluorescence in the cells was very strong at 24 h and weakened as the time increased; however, the cells maintained cy3 fluorescence for more than 72 h post-transfection (Figure 4B). To further assess the protective ability of GO-PEI complexes, we incubated naked miRNA inhibitors or GO-PEI-inhibitors (GP-inhibitors) with RNase A at 37°C. GO-PEI complexes with an N/P ratio of 30 were successfully loaded with negatively charged miRNAs and delivered miRNAs into the cells. When RNase A was added in after GP-inhibitor complex formation, the cy3-labeled miRNAs were successfully delivered into the cells without degradation, and the fluorescence of cy3 in the cells was strong. However, when RNase A was added during the formation of the GP inhibitor complex resulted in loss of the effectiveness of cy3-miRNA delivery (Figure 4C). These results demonstrated that GO-PEI could successfully load miRNA inhibitors into cells without the hydrolysis of RNA enzyme.

GP-Inhibitor Inhibits Migration and Invasion in MG63 and U2OS Cells

miR-214 was upregulated in osteosarcoma tissues and cells, miR-214 overexpression enhanced osteosarcoma cell proliferation and invasion.¹⁹ To investigate the effect of GP-inhibitors on cell migration and invasion, the MG63 and U2OS cell lines were examined by wound healing migration and transwell invasion assays after incubation with GP-inhibitor complexes. The transwell assays showed that GP inhibitor significantly reduced the invasion ability of MG63 and U2OS cells, and the relative migrated cell number dramatically decreased compared

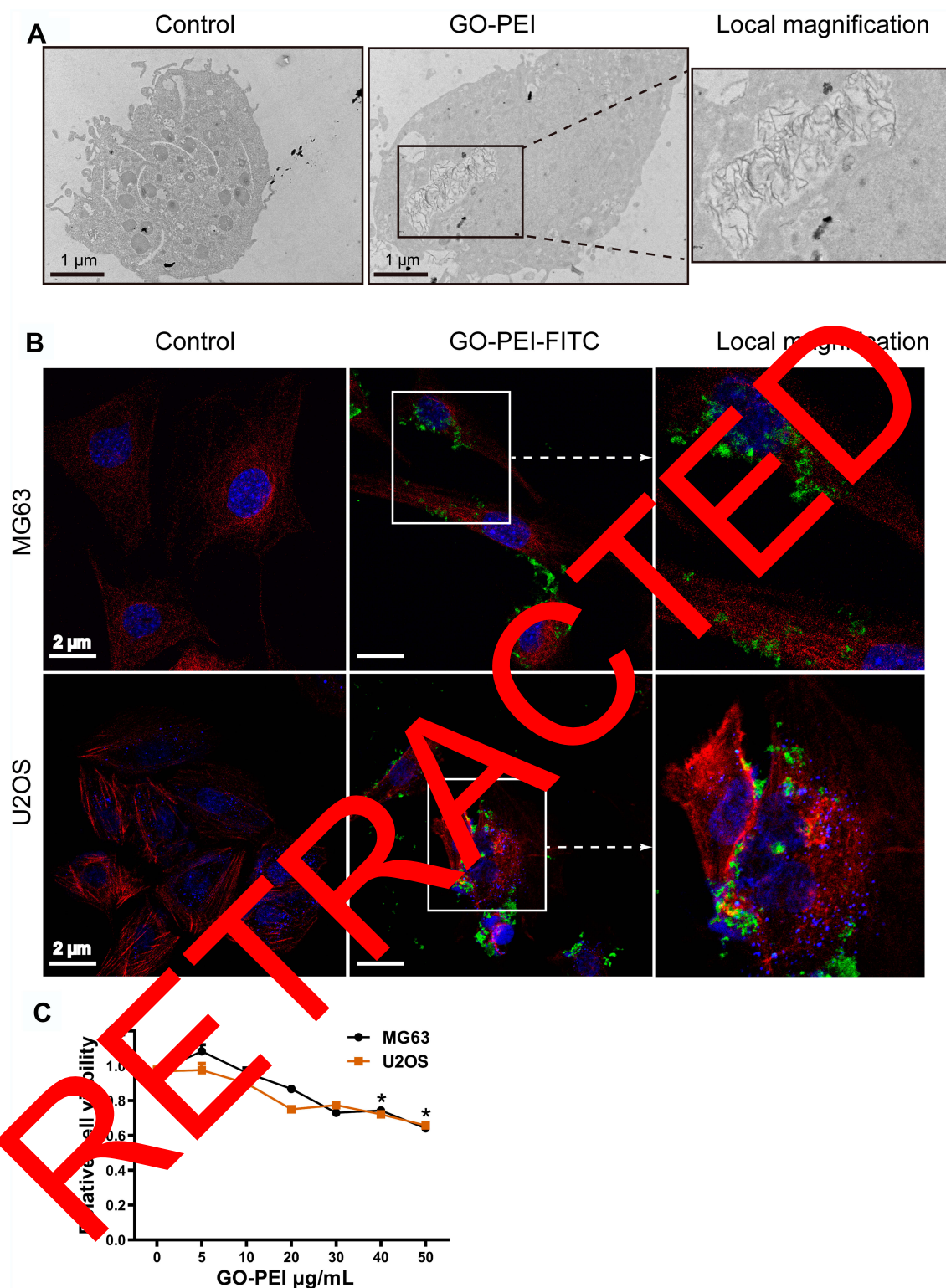


Figure 3 Uptake of GO-PEI into osteosarcoma cells. **(A)** TEM images of GO-PEI-incubated and non-incubated cells (3 $\mu\text{g/mL}$ GO-PEI in the cell culture medium). The right panel is enlarged images of white squares in the image of GO-PEI-incubated cells. Scale bars: 1 μm . **(B)** FITC-BSA-labeled GO-PEI (green) is visualized in cells through confocal laser scanning microscopy. Fluorescence images of FITC-labeled GO-PEI (green) within MG63 and U2OS cells are shown. The cell cytoskeleton was stained with α -tubulin (red) for MG63 cells and rhodamine-phalloidin (red) for U2OS cells, and the nuclei were stained with DAPI (blue). The right panel is enlarged images of white squares in the image of GO-PEI-incubated cells. Scale bars: 2 μm . **(C)** Cell viability of MG63 and U2OS cells exposed to different concentrations of GO-PEI was measured by CCK8 assays. *p < 0.05.

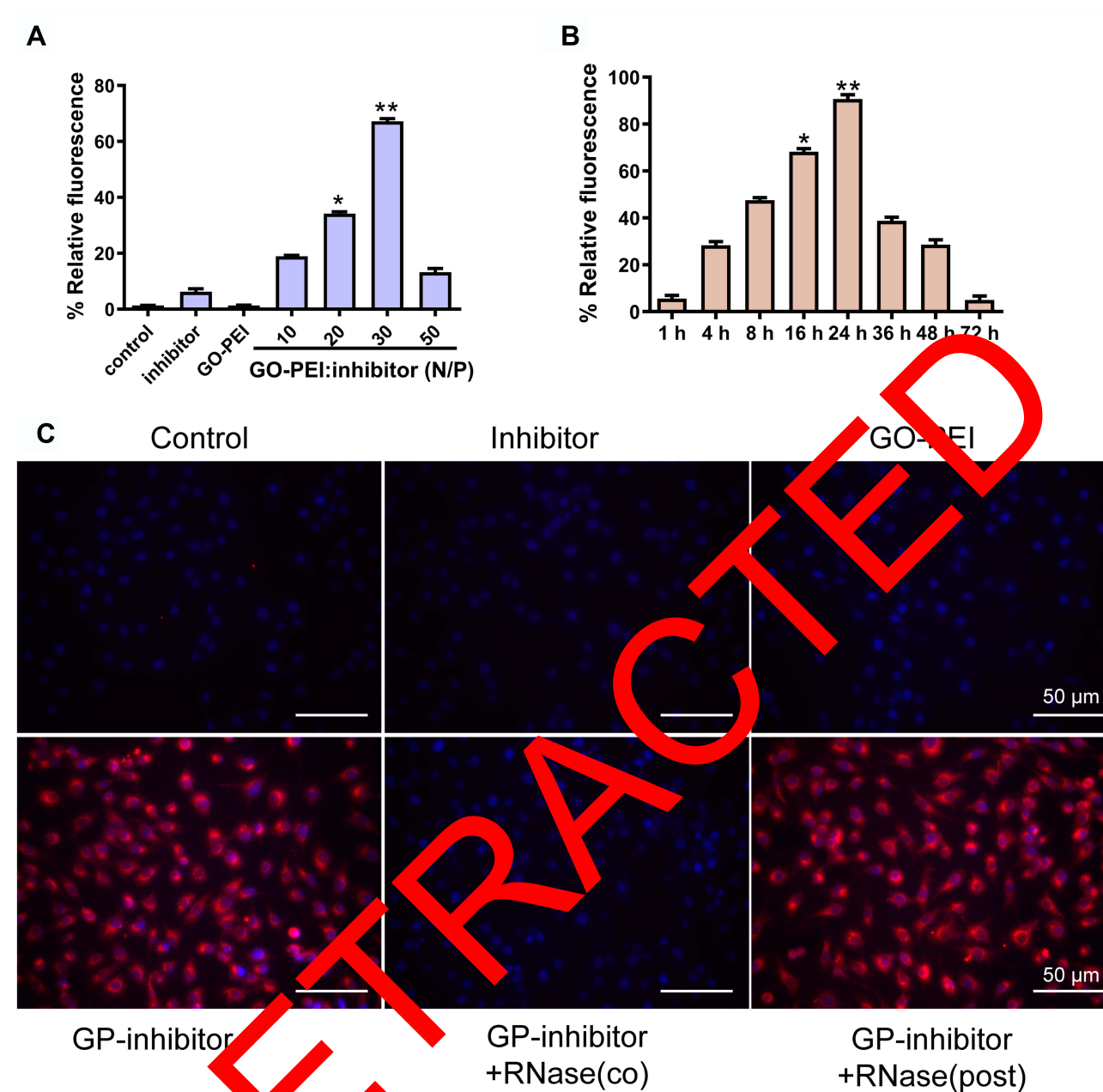


Figure 4 The efficiency evaluation of cy3-labeled miR-214 inhibitor delivery by GO-PEI complexes. **(A)** The relative fluorescence of GO-PEI and miR-214 inhibitor at various N/P ratios (1, 10, 20, 30 and 60). * $p < 0.05$, ** $p < 0.01$. **(B)** The relative fluorescence of GO-PEI and miR-214 inhibitor at the N/P ratio of 30 at different time points (1, 4, 8, 16, 24, 36, 48 and 72 h). * $p < 0.05$, ** $p < 0.01$. **(C)** Fluorescence images of cy3-labeled miR-214 (red) delivered by GO-PEI within MG63 cells are shown. The nuclei were stained with DAPI (blue). Scale bar: 50 μ m.

with that of the negative control-transfected cells and naked inhibitor-transfected cells (Figure 5A and B). Similarly, the capacity of wound healing in MG63 cells was significantly attenuated by GP-inhibitor complexes, and the relative wound area was much larger than those of the control and naked inhibitor groups after 24 h or 48 h of treatment (Figure 5C and D). Moreover, it was reported that twist was highly expressed, accompanied with N-cadherin high expressed and E-cadherin low

expressed, in osteosarcoma with metastasis.^{20,21} qRT-PCR results showed that the GP-inhibitor decreased the expression of twist and N-cadherin and increased the expression of E-cadherin compared to that of the control or the lipo-inhibitor group (Figure 5E). These results demonstrated that miR-214 has an important role in the regulation of osteosarcoma cellular motility and that the GP-inhibitor has good transfection and inhibits the invasive and metastatic capacity of osteosarcoma cells.

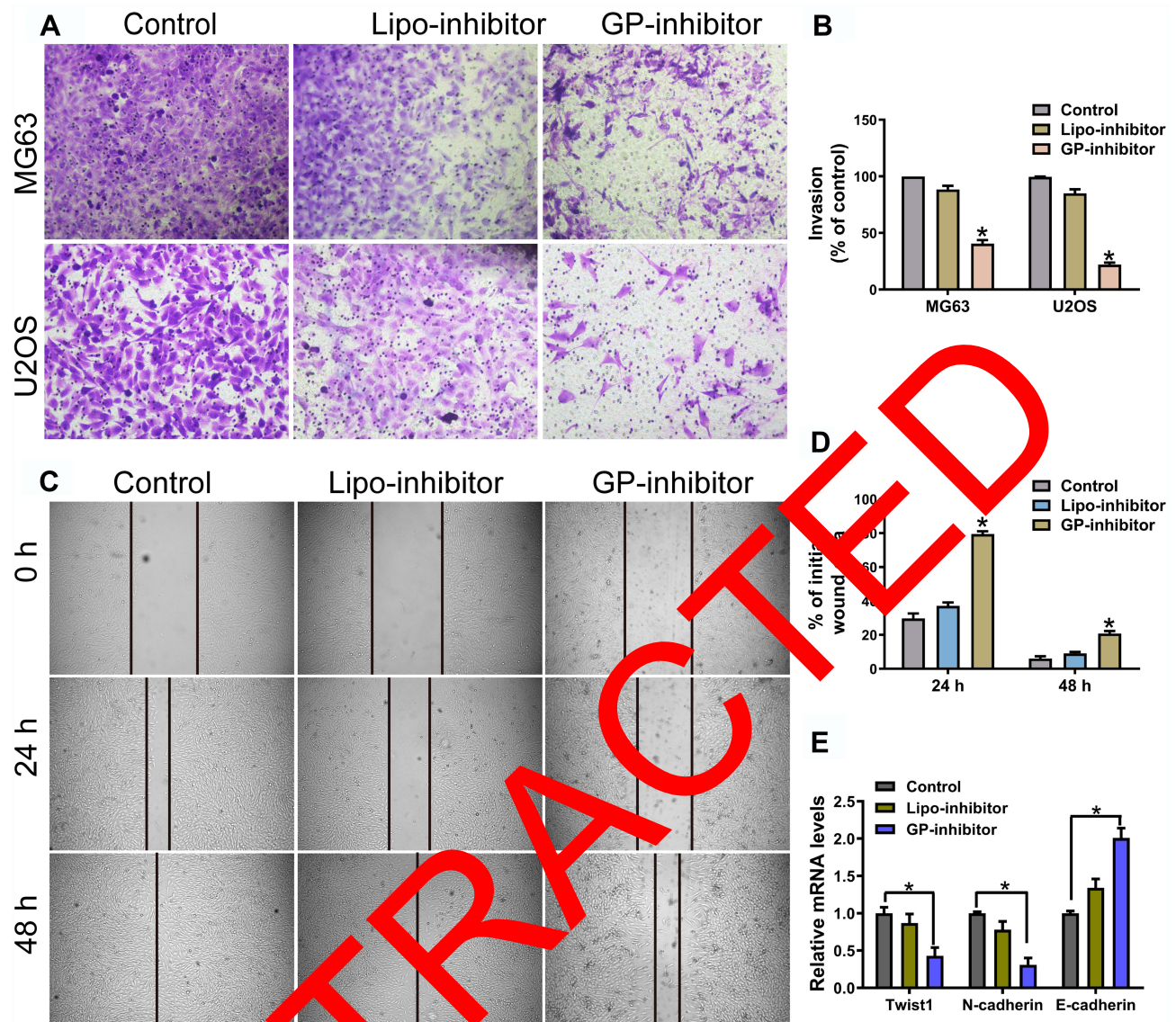


Figure 5 GP inhibitor inhibits invasion and migration in MG63 and U2OS cells. (A) The invasion of MG63 and U2OS cells were measured for 24 h using a Matrigel-coated chamber with polycarbonate filters. (B) Statistical analysis of the invasion cell ratio is presented for each group. * $p < 0.05$. (C) The wounds of confluent MG63 cells were created with 100 μ L yellow tips and incubated with a lipo-inhibitor or GP-inhibitor (5 μ g/mL). Images of the wound at different time points are shown, and the borders of the wound are shown by a pair of black lines. (D) Statistical analysis of cell migration by calculating the ratio of the final cell-free gap to the initial wound area. * $p < 0.05$. (E) The expression levels of Twist1, N-cadherin and E-cadherin after treatment with lipo-inhibitor or GP-inhibitor were evaluated by qRT-PCR. * $p < 0.05$.

GP-Inhibitor Affects miR-214-Regulated Pathways in Osteosarcoma Cells

To assess the effect of GP-inhibitor complexes on osteosarcoma cells, the level of miR-214 was analyzed by qRT-PCR. The level of miR-214 was significantly decreased by the GP-inhibitor (Figure 6A). Bioinformatic analysis showed that miR-214 binding site is highly conserved and PTEN was suggested to be a target of miR-214 in osteosarcoma cells (Figure 6B), which was in accordance with previous reports.^{19,22} miR-214 has been reported to target the 3'-UTR of PTEN and induced the activation of

the Akt and ERK signaling pathways to enhance cell growth.^{22,32,33} Thus, to analyze the underlying mechanism by which GP-inhibitors prevent osteosarcoma cell proliferation and metastasis, Western blot analysis was used to examine the effect of GP-inhibitors on the PTEN expression in MG63 cells. PTEN protein expression level was increased after GP inhibitor treatment in MG63 cells. Furthermore, GP inhibitors were found to significantly decrease the protein levels of p-Akt, and total Akt protein level was slightly decreased. In addition, the protein expression of p-ERK1/2 was significantly decreased by

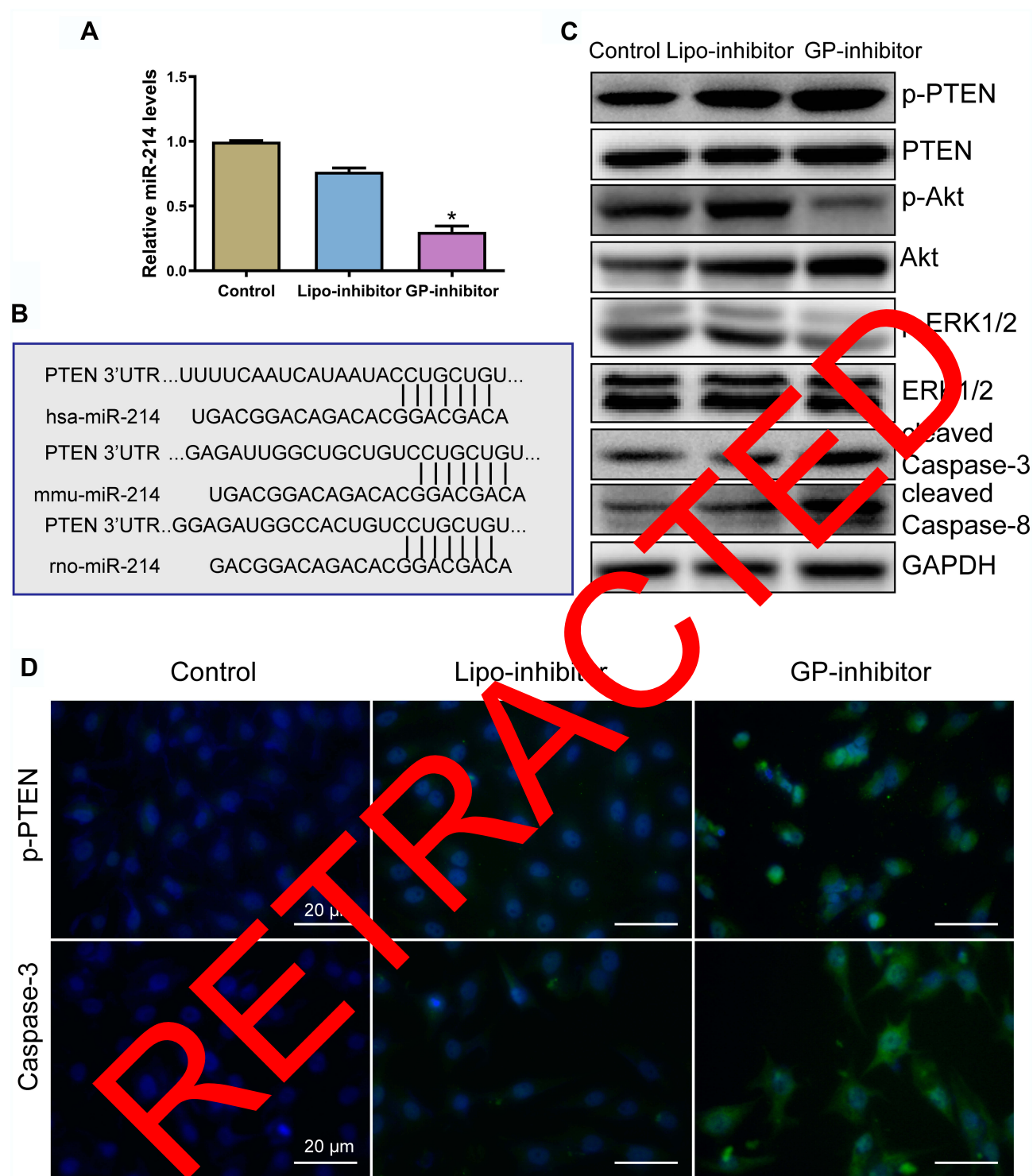


Figure 6 GP-inhibitors affected miR-214-regulated pathways in osteosarcoma cells. **(A)** Measurements of miR-214 levels in cells after treatment with lipo-inhibitor or GP-inhibitor. * $p < 0.05$. **(B)** Bioinformatic analysis revealed that miR-214 potentially targeted PTEN by the 3' untranslated region (3'-UTR), and the miR-214 binding site was conserved among different species. **(C)** The expression levels of p-PTEN, PTEN, p-Akt, Akt, p-ERK1/2, ERK1/2, cleaved caspase-3 and cleaved caspase-8 after incubation with lipo-inhibitor or GP inhibitor were evaluated by Western blotting. **(D)** Representative immunofluorescence microscopy images of p-PTEN and caspase-3 after incubation with lipo-inhibitor or GP inhibitor. Scale bars: 20 μ m.

the GP inhibitor, and total ERK1/2 protein level remained identical (Figure 6C). PTEN acts as a tumor suppressor by inhibiting PI3K and ERK1/2 signaling, activating diverse

signaling pathways involved in cell growth and cell survival.^{23–26} Furthermore, the inhibition of miR-214 by the GP-inhibitor increased the expression of caspase-3 and

caspase-8 (Figure 6C). Caspase-3 and caspase-8 are major apoptosis effectors.^{27,28} In addition, immunofluorescence staining was performed to further confirm the effect of GP-inhibitors in osteosarcoma cells. Consistent with the WB results, the expression level of PTEN was notably increased by the GP-inhibitor compared to those of the control and lipo-inhibitor groups. Accordingly, the expression of caspase-3 was also increased in GP-inhibitor-treated cells (Figure 6D). Together, these data indicated that GP inhibitors contributed to promoting apoptosis and suppressing tumor growth by targeting PTEN via PI3K/Akt- and ERK1/2-dependent signaling.

GP-Inhibitor Improved the Cytotoxicity of Cisplatin on Osteosarcoma Cells

One of the abilities of miR-214 is to inhibit apoptotic cell death and decrease chemotherapy sensitivity, which is the main cause of chemotherapy failure and disease progression.²⁹ Based on the WB and immunofluorescence results in Figure 6, GP-inhibitor complexes could efficiently increase the expression of apoptotic proteins. It was suggested that GP-inhibitors might have synergistic anticancer effects with chemotherapy. To explore whether GP-inhibitors sensitize chemotherapy, we incubated MG63 and U2OS cells for 24 h and 48 h with GP-inhibitors and cisplatin (CDDP). As shown in Figure 7A, CDDP (5 μ g/mL)-treated cells showed a slightly lower cell density than the control. However, the CDDP and GP-inhibitor co-treatment significantly decreased the cell density compared to the control. The CCK-8 assay results showed that CDDP (5 μ g/mL) imparted mild cytotoxicity compared to the untreated control. On the other hand, obvious cell growth inhibition and cell death were observed in cells co-treated with GP-inhibitor (5 μ g/mL) and CDDP (5 μ g/mL), leading to a precipitous decrease in cell viability to $28.6 \pm 1.8\%$ compared to that of the control ($82.7 \pm 1.5\%$) and GP-inhibitor ($56.33 \pm 2.2\%$) after 48 h of incubation (Figure 7B). These results indicated that the GP-inhibitor and CDDP synergistically enhanced the sensitivity of MG63 and U2OS cells.

GP-Inhibitor Showed Superior Antitumor Activities in an MG63 Xenograft Mouse Model

To assess whether GP-inhibitors could inhibit tumor growth in vivo, GP-inhibitors were used to treat tumor-bearing mice. Saline, miR-214 inhibitor, GO-PEI or GP-inhibitor

complexes (30 μ L) were injected into mice 4 times by peritumoral injection, the mice were weighed, and the tumors were measured at different time points. Representative tumor images are shown in Figure 8A. The tumors with PBS treatment increased rapidly, and the tumor volume in the naked inhibitor and GO-PEI treatment groups showed no significant difference, which suggests that the naked inhibitor and GO-PEI had a negligible effect on the osteosarcoma tumors, showing 26.9% and 20% reduction in tumor volume, respectively, compared to the PBS-treated mice. On the other hand, GP-inhibitor showed a strong effect on suppressing tumor growth (the inhibition rate was over 73.6%), with minimal change in mouse body weight during the observation period (Figure 8B and C). The mice with different treatments did not show serious body weight loss, which suggested that there was no significant toxicity from the GO-PEI and inhibitor treatment within the experimental period.

Subsequently, we removed and sectioned the tumors at day 21 for histology assessment. As shown in Figure 8D, HE staining results showed that the tumor cells were large and dense, with hyperchromatism and pleomorphism. The results illustrated that the saline and naked inhibitor treatment had no effect on cell growth in the tumor tissues, and the GO treatment did not cause cell death in tissues. Notably, the GP-inhibitor treatment-induced obvious cell death in tumor tissues. Moreover, the expression of PTEN, caspase-3 and caspase-8 proteins in tumor tissues largely increased with GP-inhibitor treatment compared to that in saline-treated tumors, which was consistent with the results in vitro. In addition, there were no noticeable signs of organ damage in the liver, spleen, lung or kidneys of mice treated with GP-inhibitor, GO-PEI or miR-214-inhibitor compared with those of the control group (Figure 9). Thus, these results indicated that GO-PEI has a latent capacity to be used as a drug delivery platform and that GP-inhibitors have a good inhibitory effect on osteosarcoma tumor progression.

Discussion

Gene therapy has been found to hold tremendous potential for the treatment of cancers beyond conventional methods.^{30–32} Nonviral vector provides safer methods without the potential side effects of viral transfection, such as immunogenicity and carcinogenesis.³³ GO has been considered an ideal vector for gene delivery.^{34,35} In this study, we demonstrated that GO-PEI was an effective nonviral carrier for miRNA delivery to suppress osteosarcoma cancer progression. GO bonded to PEI by electrostatic interactions and formed GO-PEI complexes with

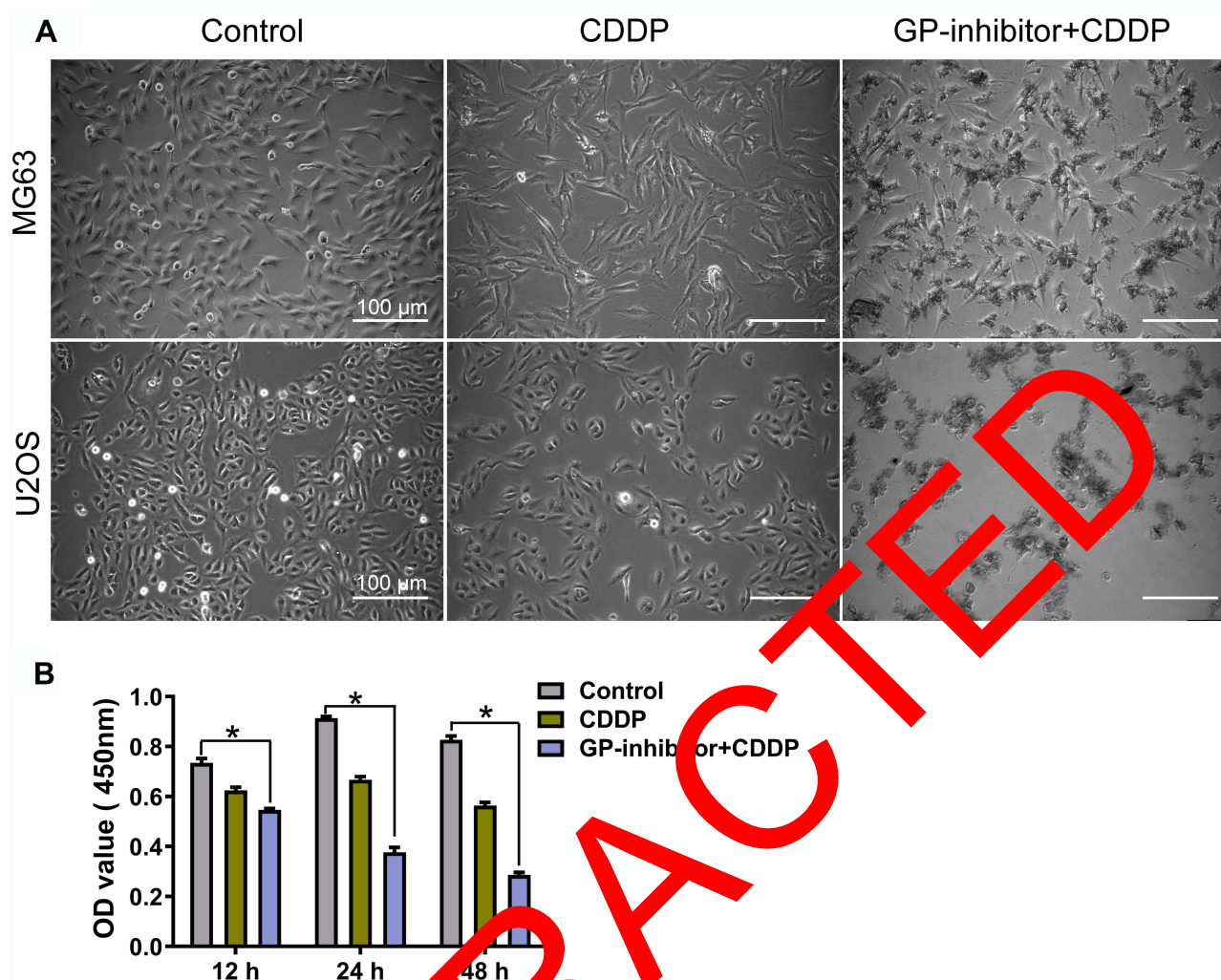


Figure 7 Combination of GP inhibitor and CDDP for improved killing of osteosarcoma cells. **(A)** Image of MG63 and U2OS cell morphology changes after co-treatment with GP-inhibitor and CDDP for 24 h. Scale bars: 100 μ m. **(B)** MG63 cell viability measured by CCK-8 assay after co-treatment with GP-inhibitor and CDDP for 12, 24 and 48 hrs. * $p < 0.05$.

a positive charge (Figure 2). PEI with a mass of cationic charge could wrap nucleic acid to protect it from RNase and facilitate the endosomal release of nucleic acid.³⁶ FITC-labeled GO-PEI was capable of endocytosis and aggregated in the cytoplasm in osteosarcoma cancer cells (Figure 3). In particular, GO-PEI exhibited high cell viabilities even at a concentration of 50 μ g/mL, likely because GO with high biocompatibility in the complexes reduced the cell cytotoxicity of PEI.

As miR-214 has been revealed to upregulate and function as oncogenic miRNA in osteosarcoma cancer,^{19,22,37} the synthetic miR-214 inhibitor might be a novel therapeutic approach for osteosarcoma cancer. In this study, we revealed that GO-PEI was capable of intracellular delivery of cy3-labeled miR-214 inhibitors and that the sequence specificity of miR-214 inhibitors was toward endogenous

miR-214; however, we found no observable fluorescence in MG63 cells treated with the naked inhibitor, GO-PEI, inhibitor or RNase co-culture (Figure 4).

miR-214 has been reported to promote osteosarcoma cancer cell invasion and proliferation.^{38,39} In this study, we found that GP-inhibitors significantly decreased osteosarcoma cells' invasion ability in transwells and migration ability after wound scratching, resulting in declining tumorigenicity. In accordance with this finding, GP-inhibitors decreased the cancer metastasis-associated genes, twist and N-cadherin, and increased E-cadherin (Figure 5). Furthermore, we investigated whether GP-inhibitors had much stronger inhibition of miR-214 expression than Lipofectamine, suggesting that miR-214 inhibition via GO-PEI effectively degraded or inhibited endogenous miR-214 (Figure 6). Research studies and biomedical database

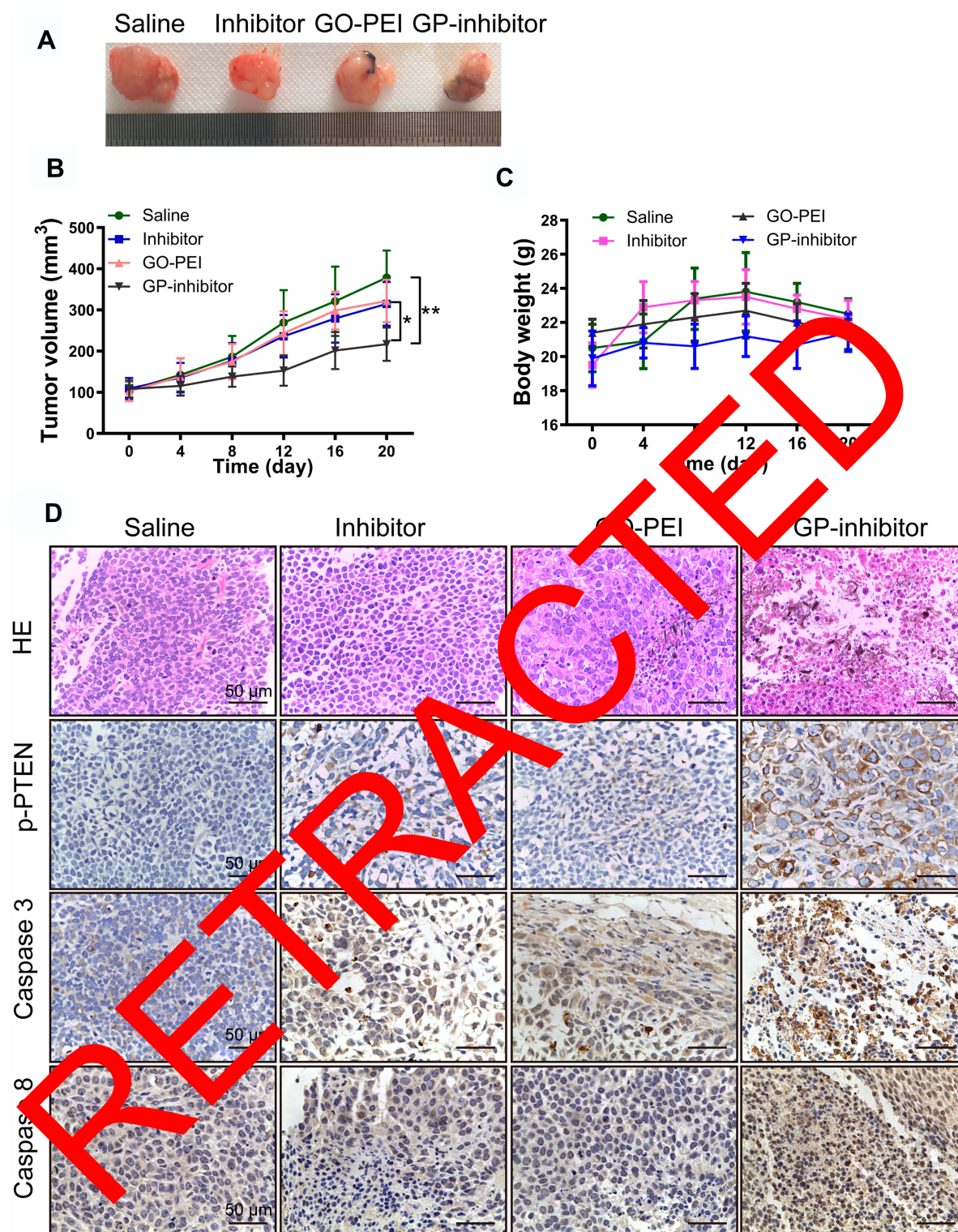


Figure 8 Antitumor activities of GP-inhibitor in an MG63 xenograft mouse model. **(A)** Representative xenograft tumor tissue images of mice treated with saline, miR-214 inhibitor, GO-PEI or GP-inhibitor complexes (30 µL). **(B)** Relative changes of tumor volume over time. The values are presented; n=5. **(C)** Relative changes of body weight over time and the values are presented; n=5. **(D)** Representative images of HE staining and immunohistochemical staining of PTEN, caspase 3 and caspase 8 in different treatment groups are shown. Scale bars: 50 µm. * $p < 0.05$; ** $p < 0.01$.

analysis demonstrated that PTEN was one of the targets of miR-214 in tumors.⁴⁰⁻⁴² A GP-inhibitor was found to significantly increase PTEN expression, likely because the

miR-214 inhibitor targeted the 3'-UTR of PTEN. Conversely, GP-inhibitors reduced Akt and ERK1/2 expression. Because PTEN is a tumor-suppressor gene that represses the

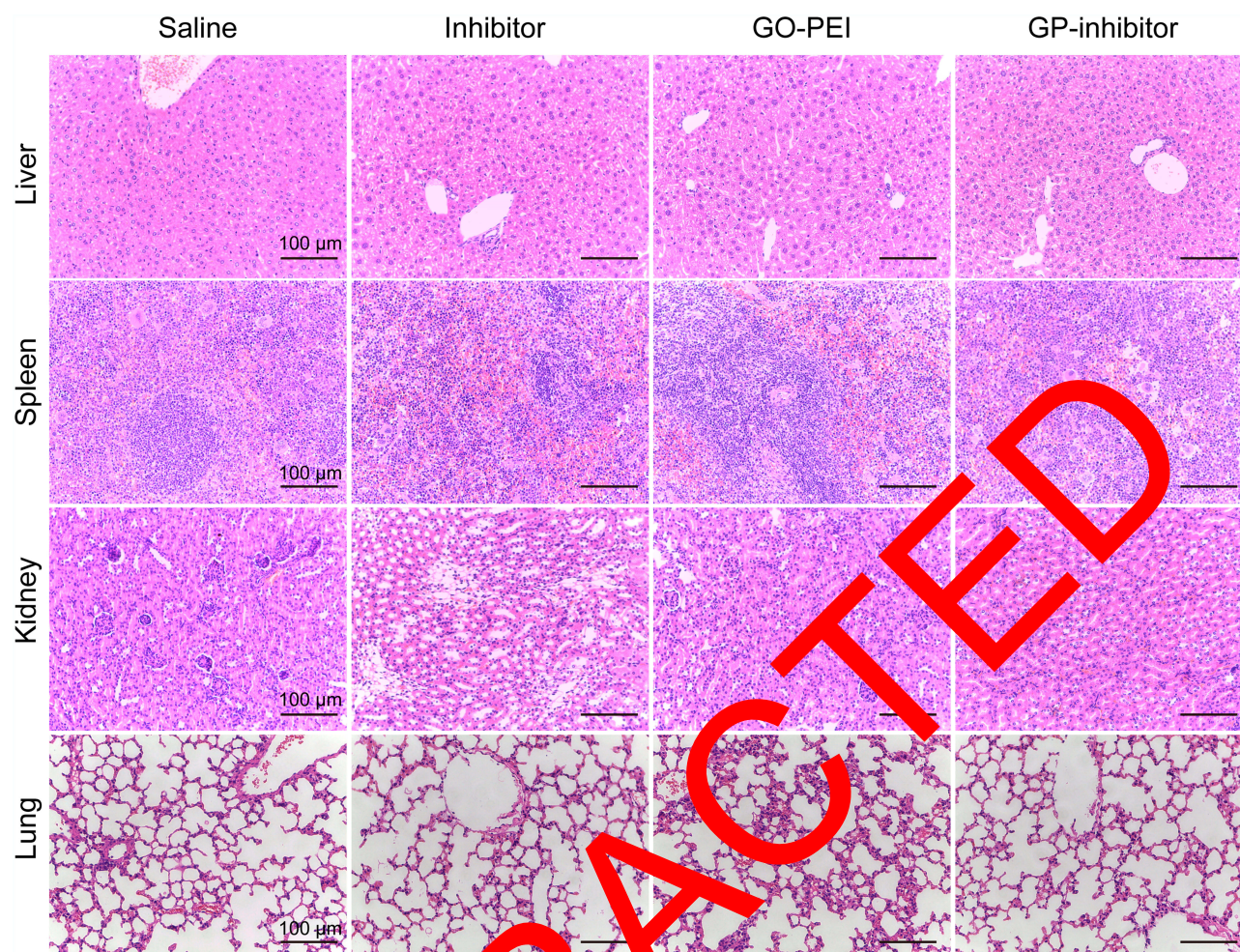


Figure 9 Organ tissue morphology observation after GP-inhibitor treatment. Histological analysis of the liver, spleen, kidney and lung after treatment with saline, miR-214 inhibitor, GO-PEI or GP inhibitor complexes (30 µL) for 20 days. Scale bars: 100 µm.

pathogenesis of various human malignancies,⁴³ PTEN can negatively regulate the PI3K/Akt²⁹ and ERK1/2 signaling pathways^{44,45} to specifically inhibit tumorigenesis and contribute to reducing twist and N-cadherin expression.^{46,47} In addition, GP-inhibitors induced apoptotic cell death and enhanced the chemotherapy sensitivity of CDDP (Figures 6 and 7). It has been reported that miR-214 was an anti-apoptotic factor in C2C12 cells,¹⁹ however, GP-inhibitors blocked the downstream effects of oncogenic miR-214 showing cell apoptosis upregulation. These results indicate that GO-PEI delivered miR-214 inhibitors that successfully degraded endogenous miR-214 and prevented the carcinogenicity of miR-214.

In the tumor xenograft models, we found that GP-inhibitor-injected mice showed obvious cancer cell death and significant tumor shrinking. GP-inhibitors not only accumulated in the tumor site but also transported into intratumoral tissues. The nanosize of GO-PEI complexes

made them easy to transport in vivo. Because high biocompatibility and stability are required for in vivo application of synthesized nanomaterials, GP-inhibitor complexes with high tumor cell targeting efficacy showed that GO-PEI had conjugation stability with miR-214 inhibitor against blood and body fluid. Here, we showed that miR-214 inhibitors could not only be delivered into osteosarcoma cells by GO-PEI but also specifically target intracellular miR-214, showing an antitumor effect.

Conclusions

High miR-214 expression in cancers has been associated with invasion, metastasis, poor prognosis and weak response to therapy.^{42,48,49} In this work, highly efficient miR-214 inhibitor delivery was shown to inhibit osteosarcoma cell invasion and migration in vitro and suppress tumor progression in vivo. This technology could deliver other therapeutic nucleic acid or nucleic acid inhibitors to treat other tumors or

diseases. Taken together, the GP inhibitor is a potential candidate for antitumor therapy in vivo.

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Disclosure

The authors report no conflicts of interest in this work.

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