

RESEARCH ARTICLE

Macrophage-secreted Exosomes Delivering miRNA-21 Inhibitor can Regulate BGC-823 Cell Proliferation

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Abstract

Exosomes, membranous nanovesicles, naturally carry bio-macromolecules or miRNA and play important roles in tumor pathogenesis. Here, we showed that macrophages cell-derived exosomes can function as vehicles to deliver exogenous miR-21 inhibitor into BGC-823 gastric cancer cells. Exosomes loaded with miR-21 inhibitor significantly increased miR-21 levels in BGC-823, but miR-21 inhibitor loaded in exosomes exerted an opposite effect. miRNA transfected with exosomes had less cellular toxicity to host cells compared to conventional transfection methods. The miR-21 inhibitor loaded exosomes promoted the migration ability and reduced apoptosis of BGC-823 gastric cancer cells. These observations indicate that miR-21 acts as a tumor promoter by targeting the PDCD4 gene and preventing apoptosis of gastric cancer cells through inhibition of PDCD4 expression. Furthermore, exosome-mediated miR-21 inhibitor delivery resulted in functionally more efficient inhibition and less cellular toxicity compared to conventional transfection methods. Similar approaches could be useful in modification of target biomolecules *in vitro* and *in vivo*. These findings contribute to our understanding of the functions of miR-21 and exosomes as a carrier for therapy of gastric cancer.

Keywords: Exosomes - gastric cancer - miR-21 - macrophages

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Introduction

Exosomes are small membranous vesicles generated by the inward budding of late endosomes, resulting in the formation of multivesicular bodies in the cytosol. They are subsequently exocytosed to the extracellular space by fusion with the plasma membrane (Vlassov et al., 2012). Exosomes are released by several cell types, including epithelial (Hu et al., 2013), dendritic (Segura et al., 2007), and tumor cells (Zech et al., 2012). Exosomes are also present in human body fluids such as amniotic fluid (Keller et al., 2011), urine (Chen et al., 2013), bronchoalveolar lavage fluid (Yamada et al., 2012), and malignant effusions (Torregrosa et al., 2012). It is possible that cell-derived vesicles such as exosomes could function as vectors for the intercellular transfer of molecules, thus providing a mechanism for cell-cell communication and the autologous amplification of cellular responses. Exosomes are involved in various pathologic conditions including tumorigenesis, establishing a pre-metastatic tumor niche, stimulating pathologic angiogenesis, promoting tumor immune escape by modulating T cell activities, and facilitating the spread of HIV (Izquierdo-Useros et al., 2010; Whiteside et al., 2011; Peinado et al., 2012; Ramakrishnaiah et al., 2012; Mu et al., 2013). Exosomes function in the transport and

intercellular delivery of endogenous proteins and nucleic acids, and can also deliver exogenous pharmaceutical proteins and nucleic acids such as mRNAs, microRNAs (miRNAs) and siRNAs (Wahlgren et al., 2012; Chen et al., 2014). Exosome secretion of microvesicles transporting miRNAs, mRNAs, and proteins through bodily fluids facilitates intercellular communication and can elicit an immune response (McDonald et al., 2014).

MiRNAs are a class of 18-27-nucleotide single-stranded RNA molecules that bind to specific target mRNAs, leading to translational repression. Specific miRNAs function as negative or positive regulators of genes involved in the development of tumor types (He et al., 2004; Bartel, 2009), such as lung cancer (Du et al., 2012), colorectal cancer (Menéndez et al., 2013), breast cancer (Liu, 2009), and gastric cancer (Wu et al., 2014). Gastric cancer is a major malignant tumor type, representing the second most common cancer and the leading cause of cancer mortality worldwide. This is especially the case in Asia, with the highest incidence being found in China, Japan, and Korea (Ferlay et al., 2010; Saeki et al., 2013), and about 1,000,000 new cases every year (Yang, 2006). There is increased expression of miR-421, miR-21, miR-221/222, and miR-106a in gastric cancer, while the expression of miR-124a, miR-

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128b, miR-148, and miR-129 is reduced (Guo et al., 2009; Konishi et al., 2012). MiRNA-21 was one of the first miRNAs to be discovered in human cells, and its expression is significantly upregulated in different kinds of solid tumors, for instance breast and gastric cancer, and is closely associated with cancer pathogenesis (Huang et al., 2013). MiR-21 expression in gastric cancer tissues is significantly higher than in adjacent tissues. Moreover, gastric cancer patients have significantly altered levels of gastric juice miR-21 compared with patients with benign gastric diseases, indicating that miR-21 is a potentially useful biomarker for gastric cancer screening (Cui et al., 2013).

Elucidation of the specific mechanisms of miR-21 function in gastric cancer and exosome-mediated miRNA delivery may provide us with the knowledge to identify promising novel treatment strategies for gastric cancer.

Materials and Methods

Cell culture

U937 human acute monocytic leukemia and BGC-823 human gastric cancer cells were purchased from the Center for Type Culture Collection of Wuhan University (Wuhan, China). U937 and BGC-823 cells were cultured in six-well plates or flasks at 37°C under 5% CO₂, in RPMI 1640-GlutaMAX medium containing 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 µg/mL amphotericin B. Differentiation of U937 cells into macrophage-like cells was induced by treatment with 0.1 mM Phorbol 12-myristate 13-acetate (PMA) for 24 h, which was purchased from Sigma Company (Missouri, USA) and dissolved in DMSO (Sigma, Missouri, USA), stored at -20°C, and used at a final concentration of 0.1 mM.

Exosome purification

U937 macrophage and BGC-823 cell culture supernatants were centrifuged at 3000 ×g for 15 min to remove cells and cell debris, and transferred to sterile tubes. The appropriate ExoQuick Exosome Precipitation Solution (System Biosciences, San Francisco, USA) was added, and tubes were mixed by inverting and then refrigerated for 30 min. The mixture was then sedimented at 1,500 ×g for 30 min, and the supernatant was removed by aspiration. Residual ExoQuick solution was removed by re-centrifugation at 1,500 ×g for 5 min followed by aspiration. Finally, exosome pellets were resuspend in 1/10 original volume of nuclease-free water, 25 µL 9% sucrose containing protease inhibitors (GenScript Biotechnology, Nanjing, China) was added, and the preparation was stored at -4°C. All procedures were carried out at 4°C.

Exosome identification by transmission electron microscopy

Freshly prepared exosomes were stained with 3% phosphotungstic acid for 5 min, placed on a copper grid, dried at 65°C, and observed at 70,000× magnification using a Hitachi H-600 transmission electron microscope (Hitachi Manufacturing Corporation, Tokyo, Japan).

Cell transfection

Exosomes were diluted 1:1 in Gene Pulser Electroporation buffer (Bio-Rad, California, USA). MiR-21 inhibitor, miR-21 mimics, or negative control miRNA (Zimmer Pharmaceutical Biotechnology, Shanghai, China) was added to a final concentration of 150 pmol to a 1 µg/µL exosome sample. The mixture was transferred to a 0.2 cm electroporation cuvette and electroporation was performed at 0.15 kV and 100 µF. Finally, exosomes were treated with 1 U RNase H to degrade miRNA outside the exosomes and then re-isolated using ExoQuick solution (System Biosciences, San Francisco, USA).

Co-culture experiments

Exosomes loaded with miR-21 inhibitor, miR-21 mimics, or negative control miRNA (Zimmer Pharmaceutical Biotechnology, Shanghai, China) were co-cultured with macrophages for 6 h, followed by washing and medium replacement. The level of miR-21 was then assessed after 24 h. U6 was used as internal control for RT-PCR analysis. For miR-21 inhibition, macrophages were seeded 1 day before treatment, and then subjected to different treatments for 24 h. Treatments included miR-21 inhibitor-loaded exosomes, negative miRNA-loaded exosomes, miR-21 mimic-loaded exosomes, miR-21 inhibitor plus ImagenFect RNAi transfection kit (Naomi Biotech, Wuxi, China), and miR-21 inhibitor plus X-tremeGENE HP RNAi transfection kit (Roche, Basel, Switzerland). The same miR-21 inhibitor concentration (300 pmol) was used for both exosome loading and chemical transfection methods. ImagenFect RNAi and X-tremeGENE HP RNAi transfection kits were used according to the manufacturer's recommendations. Relative miR-21 expression was measured by quantitative PCR (qPCR) using SYBR Premix ExTaq (TaKaRa Company, DaLian, China) according to the manufacturer's instructions. PCR cycling conditions were 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min, for 30 cycles. Differences in relative expression between normal and pericarcinoma and gastric cancer tissues were calculated using the 2^{-ΔΔCt} method, as follows: $\Delta\Delta Ct = \Delta Ct_{\text{reference}} - \Delta Ct_{\text{sample}}$, where ΔCt is the difference in the cycling threshold between the gene of interest and the U6 "housekeeping" gene, $\Delta Ct_{\text{sample}}$ is the Ct value for a given sample of pericarcinoma or gastric cancer normalized to the U6 gene, and $\Delta Ct_{\text{reference}}$ is the Ct value corresponding to control samples normalized to U6 expression. Duplicate samples were run and mean Ct values were calculated.

PCR analysis

Total RNA was extracted using Trizol (TianGen Biotech, Beijing, China), according to the manufacturer's instructions. Reverse transcriptase reactions contained a 50 ng RNA sample 1 µL, 0.5 µL each forward and reverse primers, 4 µL 1×RT buffer (GenScript, Nanjing, China), 2 µL 0.25 mM dNTPs, 0.5 µL 3.33 U/ml MultiScribe reverse transcriptase (GenScript), and 0.5 µL 0.25 U/ml RNase inhibitor (GenScript) and 11 µL DEPC water. Reactions were incubated in an Eppendorf Realplex2 Detection System (Eppendorf, Germany) for 30 min at 16°C, 30

min at 42°C, and 5 min at 85°C, and then at a constant temperature of 4°C. Reaction conditions for qPCR were 35 cycles of 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min.

For RT-PCR, 20 µL reactions containing 1 µL cDNA, 0.5 µL each forward and reverse primers, 2 µL 2.5 mM dNTPs (GenScript), 0.5 µL DNA polymerase (10 U/µL) (GenScript Company, Nanjing, China), 4 µL 1× buffer (GenScript) and 11.5 µL DEPC water were subjected to 35 cycles of 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min. PCR products were analyzed by 2.5% agarose gel electrophoresis. Specific primers were: miR-21 forward, 5' -ACGTTGTGTAGCTTATCAGACTG-3', miR-21 reverse, 5' -AATGGTTGTTCTCCACACTCTC-3'; U6 miR forward, 5' -ATTGGAACGATACAGAGAAGATT-3, U6 miR reverse 5' -GGAACGCTTC

ACGAATTTG-3'; miR-21 mimics, UAGCU UAUCAGACUGAUGUU and GA/AACAUCA GUCUGAUAAGCUAAU; miR-21 inhibitor, UCAACA UCAGUCUGAUAAGCUA; negative miRNA, AACC GGTATCGACCGAACAAG.

Western blotting analysis of PDCD4

Proteins were extracted from BGC-823 cells transfected with various siRNA-loaded exosomes using a protein extraction kit (Tiangen Biotech, Beijing, China), and protein concentrations were measured by the Bradford method. Protein samples (50 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Massachusetts, USA). Membranes were incubated with a rabbit anti-mouse PDCD4 antibody (Kuang Bo Biotechnology, Beijing, China), and then with an anti-rabbit horseradish peroxidase-labeled IgG secondary antibody.

Cell migration and invasion assay

Cell migration assays were performed using transwell chambers with 8 µm pores (Chemicon, California, USA). Culture medium (500 µL) containing 10% FBS (as a chemoattractant) was added to the lower chamber of each well. Cell suspension (106 cells, 300 µL) was added to the upper chamber and plates were incubated at 37°C for 24 h. Cells that migrated to the lower surface of the polycarbonate membrane were stained with Giemsa solution and quantified by counting five randomly selected microscope fields at 40× magnification. Cell invasion assays were performed in a similar way using transwell chambers coated with Matrigel, and the number of cells that passed through the Matrigel matrix was determined after 24 h.

Flow cytometry analysis

BGC-823 cells were transfected with exosomes loaded with various siRNAs, incubated for 24 h, and then harvested and washed twice with PBS containing 0.2% BSA. Apoptosis was quantitatively determined in transfected BGC-823 cells by flow cytometry using an annexin V-FITC/PI apoptosis detection kit according to the manufacturer's instructions (BD, New Jersey, USA). Cell analysis and sorting were performed using

a fluorescence-activated cell sorter (Beckman MOFLO XDP, California, USA).

Statistical analysis

Within each experiment, triplicates were performed for each condition or group. Data were analyzed using SPSS 16.0 (International Business Machines Corporation, New York, USA) and are presented as means ± SEM of at least three independent experiments. Differences within each group were subjected to a t-test or q-test. Statistical significance was set at $P \leq 0.05$ between groups (indicated by an asterisk).

Results

Exosome morphology and composition

Exosomes obtained from macrophages or BGC-823 cells were observed by phosphotungstic staining and transmission electron microscopy. This revealed a homogenous population of morphologically typical vesicles 30-100 nm in diameter, as analyzed by NanoSight statistical software (Figure 1A-C). These were similar in appearance and size to those in published reports (Delcayre et al., 2006). Additionally, electrophoretic analysis showed that exosomes from both macrophages and BGC-823 cells contain proteins of many different sizes (Figure 1D), although there are clearly many different protein components in exosomes from macrophages (exo M) and from BGC-823 cells (exo B).

Analysis of miR-421 levels and exosome-based delivery leads to minimal cytotoxicity

miR-21 expression in macrophage or BGC-823 cell exosomes and in whole cells was investigated by qPCR (Figure 2A). miR-21 mimics, miR-21 inhibitor, and negative miRNA were transported into BGC-823 cells by exosomes secreted from macrophages, and miR-21 levels in each experimental group were determined by

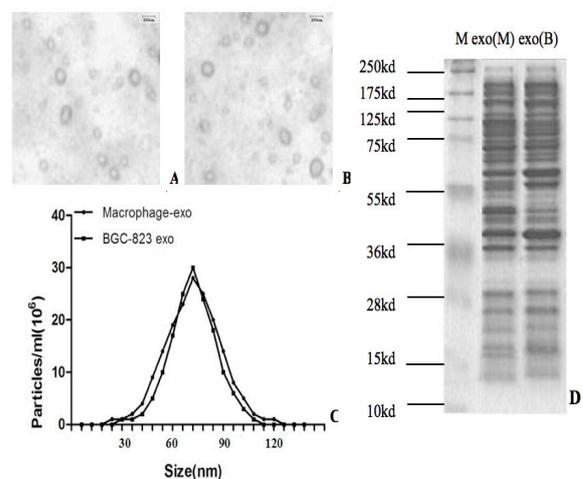


Figure 1. Analysis of Exosome Morphology and Composition. A or B: Exosomes were observed by transmission electron microscopy (magnification $\times 70,000$), scale bar: 100 nm; C: The diameter of exosomes was determined using NanoSight statistical software. D: Protein expression in exosomes secreted from macrophages and BGC-823 cells was analyzed by 10% SDS-PAGE

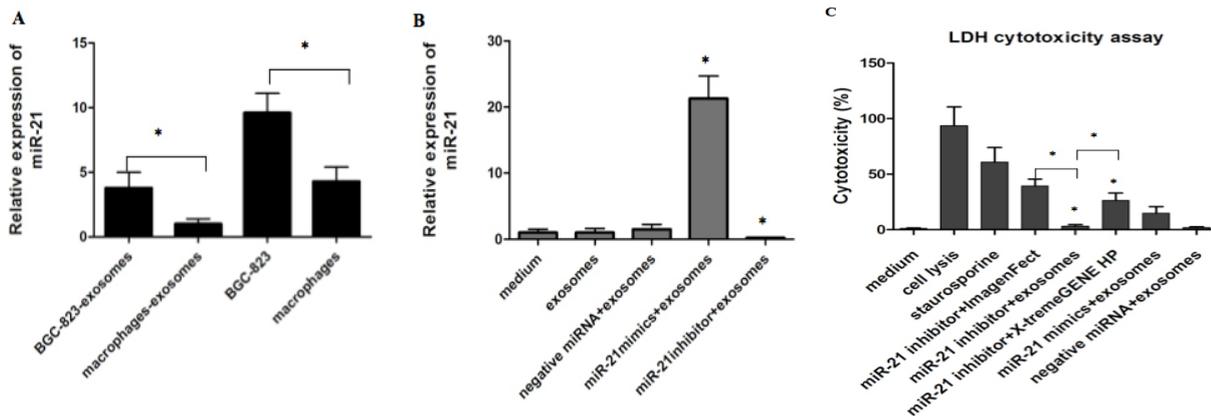


Figure 2. Analysis of miR-21 Levels in Exosomes, Exosome-secreting Cells, and Cells Transfected with siRNA-loaded Exosomes. Meanwhile Cytotoxicity evaluation of exosomes-based miR-21 inhibitor delivery and conventional transfection reagents. **A)** MiR-21 expression in exosomes secreted from macrophages or BGC-823 cells was analyzed by qPCR. **B)** miR-21 levels in BGC-823 cells transfected with miR-21 mimics, miR-21 inhibitor, or negative miRNA loaded into exosomes secreted from macrophages. miR-21 levels were normalized to endogenous GAPDH (mean ± SEM, three independent experiments). Statistical analysis was performed using SPSS 16.0, * $P < 0.05$. **C)** After 24 h of co-culture, miR-21 inhibitor-loaded exosomes showed minimal toxicity, while miR-21 inhibitor plus ImagenFect or X-tremeGENE HP showed significant cytotoxicity (* $P < 0.05$). Statistical analysis was performed using SPSS 16.0, and all results represent three independent experiments

qPCR (Figure 2B). There was significantly less miR-21 in exosomes than in macrophages or BGC-823 cells. Importantly, the lowest miR-21 expression level was observed in exosomes secreted from macrophages. miR-21 expression was lower in BGC-823 cells transfected with miR-21 inhibitor-loaded exosomes, but higher in BGC-823 cells treated with miR-21 mimic-loaded exosomes.

The cytotoxicity of conventional transfection reagents toward BGC-823 cells was analyzed by measuring lactate dehydrogenase (LDH) expression. The LDH level and percentage cytotoxicity were similar in BGC-823 cells transfected with miR-21 inhibitor-loaded exosomes following transfection with two different reagents (ImagenFect and X-tremeGENE HP; Figure 2C). In all three experimental groups, LDH expression and percentage cytotoxicity increased significantly ($P < 0.05$) after 24h.

Analysis of PDCD4 expression

BGC-823 cells were transfected with miR-21 mimics, miR-21 inhibitor, or negative siRNA loaded into exosomes and secreted from macrophages. Following this, a potential miR-21 target gene, programmed cell death protein 4 (PDCD4), and the encoded protein, which is thought to play an important role in apoptosis, were analyzed by RT-PCR and western blotting, respectively (Figure 3). Levels of PDCD4 gene and protein expression in BGC-823 cells were significantly increased following transfection with miR-21 inhibitor plus exosomes, but were significantly reduced following transfection with miR-21 mimics plus exosomes.

Cell migration assay

MiR-21 mimics, miR-21 inhibitor, or negative miRNA was transfected into BGC-823 cells via exosomes secreted from macrophages, and cells from the different experimental groups were incubated in transwell

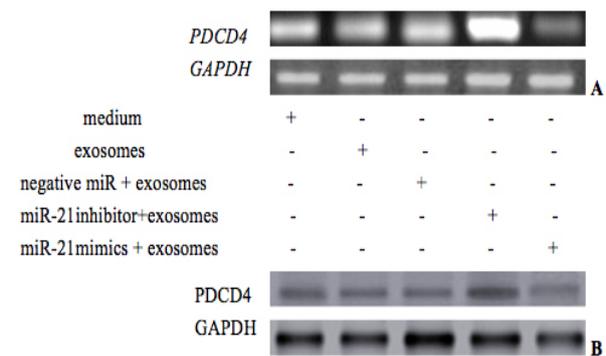


Figure 3. Analysis of PDCD4 Gene and Protein Levels in BGC-823 Cells Transfected with Various siRNAs Loaded into Exosomes. **A)** RT-PCR analysis of PDCD4 mRNA; **B)** Western blotting analysis of PDCD4 proteins. Data represent three independent experiments

chambers for 24 h. Migrated cells were stained with Giemsa and observed by microscopy (Figure 4A). Cells of all four groups migrated through the membrane, but significantly fewer ($P < 0.05$) cells transfected with miR-21 inhibitor plus exosomes had migrated compared with all other groups. These data indicate that reduced miR-21 expression inhibits BGC-823 cell migration.

Changes that occur in the plasma membrane during apoptosis can be detected by annexin-V staining. We therefore measured apoptosis in siRNA-transfected BGC-823 cells using annexin-V FITC and PI staining and flow cytometry (Figure 4C). BGC-823 cells transfected with miR-21 mimic-loaded exosomes had very low levels of apoptosis; in contrast, in BGC-823 cells transfected with miR-21 inhibitor-loaded exosomes there was a clear induction of apoptosis or necrosis. These results indicate that miR-21 may function as a tumor promoter in BGC-823 gastric cancer cells.

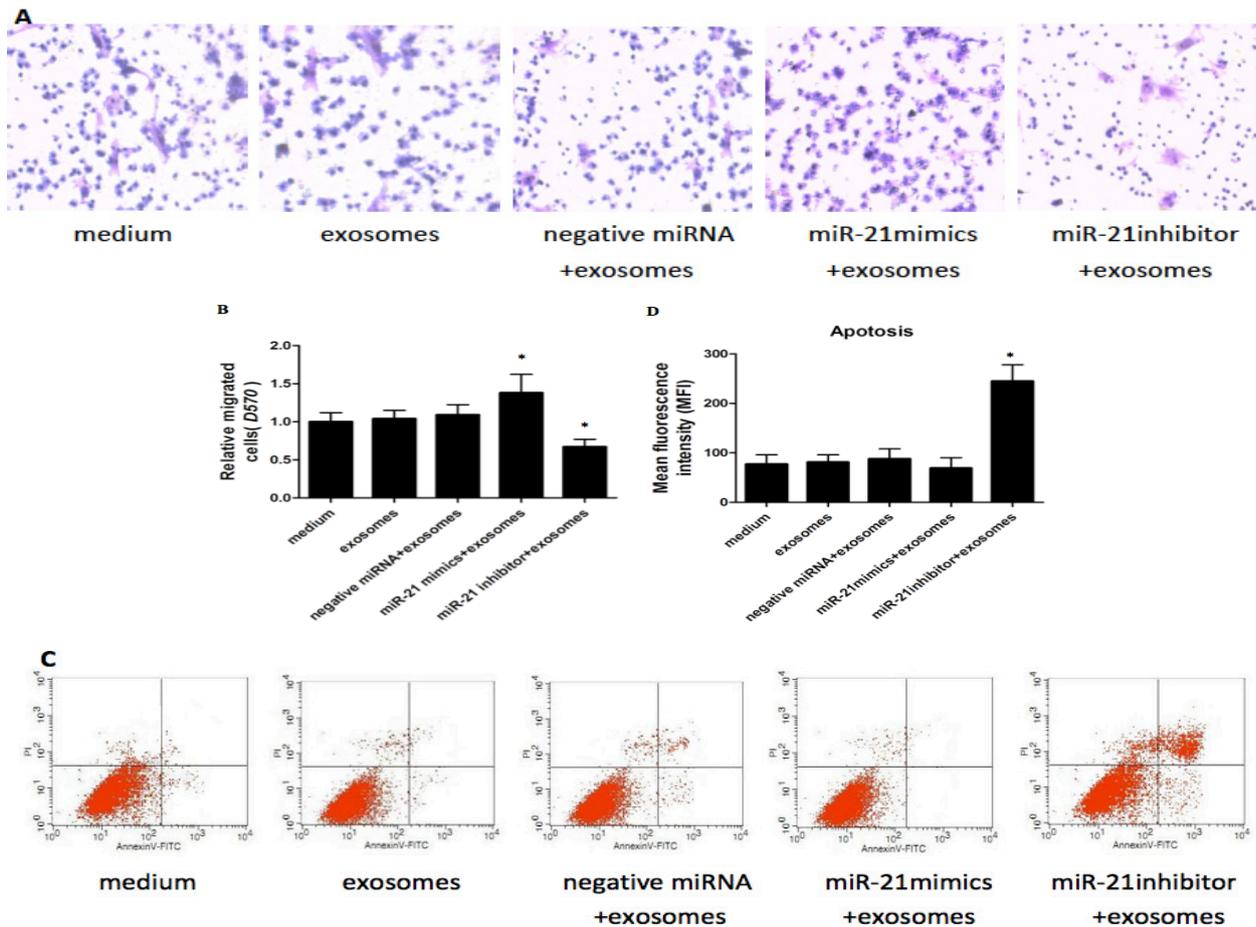


Figure 4. Analysis of the Migration Capacity of BGC-823 Cells Transfected siRNA Loaded Into Exosomes (magnification $\times 40$), and flow Cytometric Determination of Apoptosis Levels in BGC-823 Cells Transfected with miR-21 mimics or miR-21 inhibitor Loaded Into Exosomes. A) Giemsa staining of migrated BGC-823 cells transfected with siRNA plus exosomes; B) D570 values were compared among different treated groups using SPSS16.0 statistical software (* $P < 0.05$). Data represent three independent experiments. C) Flow cytometric analysis; D) Quantification of apoptosis and necrosis using SPSS 16.0. Data are presented as means \pm SEM ($n = 3$ for three independent experiments), * $P < 0.035$

Discussion

Exosomes are natural nano-sized membranous vesicles and represent a hot topic in nanomedical research. As a consequence of their nanoscale size, their potential ability to express targeting ligands and deliver biomacromolecules, and their strong biocompatibility, exosomes are now emerging as attractive biological nanovesicle platforms for loading and carrying biomacromolecules for nanomedicine applications (Kooijmans et al., 2012; Zeng et al., 2002).

Exosomes secreted from most tumor cells, including breast cancer, gastric cancer, and lung cancer cells, contain a variety of miRNAs. MiRNAs are regulatory non-coding RNAs that control the activity of protein-coding genes by combining with the 3' UTR of target mRNAs and thereby inhibiting gene expression at the transcriptional or post-transcriptional level. They are involved in various processes from early developmental processes to cell differentiation and apoptosis. Multiple miRNAs associated with gastric carcinogenesis display tissue-specific expression, and can function as either tumor suppressors or oncogenes. A number of specific miRNAs are differentially expressed in gastric cancer and normal gastric mucosa: miR-21, miR-34b/c, and

miR-221/222 are upregulated gastric tumors, whereas miR-124a, miR-128b, and miR-148 are downregulated (Li et al., 2012; Hood et al., 2012). A clinical study found high miR-21 levels in exosomes from ovarian carcinoma effusion supernatants by qPCR, and these were associated with poor progression-free survival times (Vaksman et al., 2014). Additionally, human bronchial epithelial (HBE) cells release exosomes containing miR-21 that can stimulate proliferation in neighboring normal HBE cells. These data thus support the concept that exosomal miRNAs are involved in cell-cell communication during carcinogenesis induced by environmental chemicals (Xu et al., 2014). We found miR-21 to be expressed in U937 macrophages and BGC-823 cells, and in exosomes secreted from both cell types. However, expression levels were low in exosomes, especially in those secreted from macrophages, compared with expression in whole cells.

Exosomes have several characteristics that make them suitable nano-vehicles: they are small, relatively homogenous, and stable. Moreover, they can mediate gene delivery without inducing adverse immune reactions and pro-inflammatory responses (Alvarez-Erviti et al., 2011). Conversely, many of the frequently used gene therapy methods, including viral vectors, liposomes, and lipid nanoparticles that activate the host immune system, induce

toxicity, and trigger inflammatory responses (Zhuang et al., 2011; Seow et al., 2009; Marcus et al., 2013). In this study, exosomes carrying a miR-21 inhibitor into BGC-823 cells had toxic effects on host cells, in contrast to the conventional ImagenFect and X-tremeGENE HP transfection reagents. Furthermore, miR-21 expression was significantly reduced in BGC-823 cells transfected with exosomes loaded with miR-21 inhibitor. In contrast, miR-21 expression was significantly increased in BGC-823 cells transfected with miR-21 mimic-loaded exosomes. These data suggest that exosomes represent a good system for carrying drugs into target cells to treat diseases.

Antisense inhibition of miR-21 in K562 cells suppressed cell migration, promoted cell apoptosis, inhibited cell growth, and upregulated the PDCD4 tumor suppressor gene. Additionally, treatment with pre-miRNA-21 increased cell migration and decreased apoptosis without affecting cell proliferation. These data suggest that miR-21 plays an oncogenic role in chronic myeloid leukemia (CML) and that antisense inhibition of miR-21 may therefore be therapeutically useful for CML (Hu et al., 2010). MiR-21 overexpression significantly enhanced proliferation or invasion and inhibited apoptosis in TPC-1 cells. Additionally, miR-21 may play an oncogenic role by directly targeting PDCD4 in the development of papillary thyroid carcinoma (PTC) (Zhang et al., 2012). We transfected miR-21 inhibitor into BGC-823 cells via exosomes secreted from macrophages, and found a clear inhibition of cell migration and a remarkable increase in apoptosis.

In this study, our findings indicate that miR-21 up-regulation in gastric cancer contributes to cell proliferation, thus suggesting that miR-21 may be a therapeutic target for gastric cancer. Additionally, exosomes secreted from macrophages may be efficient vehicles for RNA-based therapeutic strategies. We provided proof of concept for the use of exosomes as efficient delivery nano-vehicles with minimal cytotoxicity. We have therefore further elucidated the molecular mechanism associated with gastric cancer initiation and progression.

Acknowledgements

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