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Exosome-formed synthetic microRNA-143 is transferred to osteosarcoma cells and inhibits their migration

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ABSTRACT

MicroRNAs (miRNAs) have emerged as potential anticancer agents, but their clinical application is limited by the lack of an effective delivery system to tumors. Exosomes are small vesicles that play important roles in intercellular communication. Here, we show that synthetic miR-143 introduced into cells is released enveloped in exosomes and that the secreted exosome-formed miR-143 is transferred to osteosarcoma cells. The delivery of exosome-formed miR-143 significantly reduced the migration of osteosarcoma cells. The delivery efficiency of exosome-formed miR-143 was less than that achieved with lipofection, but the migratory potential of osteosarcoma cells was similarly inhibited after both strategies. Our results suggest that exosomes can deliver synthetic miR-143 and are a potentially efficient and functional delivery system.

1. Introduction

Osteosarcoma is a primary malignant bone tumor predominantly affecting children and adolescents, and accounting for approximately 60% of malignant bone tumors [1]. Major advances in treatment over the past three decades, especially the introduction of surgery combined with neoadjuvant and adjuvant chemotherapy, have markedly improved patient outcomes. However, the survival of patients with metastatic disease is less than 30% [2]. Further efforts to improve patient outcomes, including novel treatment protocols, have not significantly affected the overall or disease-free survival of osteosarcoma patients over the past 20 years [3]. The suppression of metastasis is critical for the reduction of mortality associated with osteosarcoma. Therefore, new alternative therapeutic interventions are required to prevent the metastasis of osteosarcoma.

MicroRNAs (miRNAs) are small noncoding RNAs of 18–25 nucleotides that repress the translation of and/or cleave mRNAs by partially base-pairing with the untranslated regions and/or coding regions of their target transcripts [4,5]. Therefore, miRNAs can silence the expression of multiple genes. It is estimated that miRNAs potentially regulate at least 20–30% of all human genes [6,7]. miRNAs have been shown to regulate various critical biological processes, including cell differentiation, apoptosis, and cell proliferation, and their dysregulated expression causes several diseases, including cancer and arthritis, by disrupting the regulatory networks of their target genes [8,9]. Therefore, miRNAs are potential therapeutic targets for several diseases. There are two therapeutic approaches to those diseases attributable to abnormally regulated miRNA: by restoring downregulated miRNAs by supplementation with miRNA mimics, or by blocking upregulated miRNAs with their antisense miRNAs. miR-143 is the most frequently downregulated miRNA in the 143B human osteosarcoma cell line, which is highly metastatic to the lung [10,11]. The systemic administration of miR-143 with atelocollagen into a mouse model of cancer suppressed the spontaneous lung metastasis of osteosarcoma [10]. The restoration of miR-143 expression significantly reduced cell viability, promoted cell apoptosis, and suppressed tumorigenicity [11]. It has been suggested that the restoration of miR-143 represents a promising therapeutic strategy for osteosarcoma, but nucleic acid therapies using small interfering RNA (siRNA) or miRNA have not yet been established because no delivery system is available. This is the most significant problem for their clinical application.

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Although many studies have focused on the intracellular miRNAs, miRNAs have also recently been found in extracellular compartments, including the blood and other body fluids [12]. It has been reported that miRNAs packaged in extracellular vesicles, such as exosomes, are secreted from cells. Exosomes are small vesicles (30–100 nm) derived from the membranes of multivesicular bodies and constitutively released when the multivesicular bodies fuse with the cell membrane [12–15]. Therefore, the extracellular miRNAs in exosomes represent genetic material that is transferable from tissue to tissue, and thus constitute new communication factors [12,13]. Increasing our understanding of exosomes and extracellular miRNAs should clarify this new communication network, which functions under many physiological and pathophysiological conditions, and should also offer new ways to treat various diseases. Several studies have demonstrated the delivery of siRNAs and miRNAs using exosomes as carrier [16,17].

In this study, we show that exosome-formed miR-143 was secreted when the synthetic double-strand miRNA was introduced into mesenchymal stem cells (MSCs), and was easily transferred into osteosarcoma cells, suppressing their migration. These results suggest that exosomes are an efficient delivery system for exosome-formed miRNAs.

2. Materials and methods

2.1. Cell culture

The human osteosarcoma cell line 143B was obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) with 10% fetal bovine serum (FBS; Life Technologies, NY) and 1% antibiotic–antimycotic solution (Nakalai Tesque, Kyoto, Japan). Human bone-marrow-derived MSCs were obtained from Lonza and cultured using Mesenchymal Stem Cell Growth Medium BulletKit (Lonza, Basel, Switzerland).

2.2. Transfection assays

143B cells (1×10^5) or MSCs (5×10^4) were seeded into the wells of 12-well plates and incubated for 3 or 24 h. Synthesized RNA oligonucleotides 5′- GGUGCAGUGCUGCAUCUCUGG-3′ and 5′-UGAGAUGAAGCACUGUAGCUC-3′ were annealed to generate double-stranded miR-143 (Hokkaido System Sciences, Hokkaido, Japan). The cells were transfected with miR-143 or silencer negative control#2 RNA (siNega; Life Technologies), using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies).

2.3. Preparation of conditioned medium and exosomes

MSCs (5×10^4/ml) were seeded into the wells of a 12-well plate and transfected with miR-143 or siNega. Before the culture medium was collected, the MSCs were washed three times with DMEM supplemented with 10% FBS, and the medium was switched to fresh medium without FBS. After incubation for 24 h, the medium was collected and centrifuged at 2380*g* for 15 min at room temperature to thoroughly remove any cellular debris. The supernatant was used for miRNA extraction, functional assays, and exosome isolation. For exosome preparation, the conditioned medium (1 ml) was ultracentrifuged at 110,000*g* for 70 min at 4 °C using a Beckman Optima™ XL-80K Ultracentrifuge. The supernatant (exosome-depleted conditioned medium) fraction was removed and the pellet was resuspended in phosphate-buffered saline (PBS) and centrifuged at 110,000*g* for 70 min at 4 °C. The pellet was finally resuspended in 50 μl of PBS. The exosomes (50 μl) were used for miRNA extraction and functional assays and the supernatant fraction (about 1 ml) as the exosome-depleted conditioned medium.

2.4. miRNA extraction and quantitative real-time PCR

Total cellular RNA was extracted with TRIzol Reagent (Life Technologies). The extracellular miRNAs were isolated with the mirVana miRNA Isolation Kit (Life Technologies). The levels of extracellular miRNAs were determined in a fixed volume (1000 μl) of conditioned medium and 5 μl of synthetic cel-miR-39 was added to each aliquot. miR-143 was amplified with real-time PCR using the TaqMan MicroRNA Assay (Life Technologies). Cel-miR-39 or RNU6B was used as the invariant control for conditioned medium or cells, respectively. The ΔΔCt method was used to analyze the real-time PCR data.

2.5. Cell proliferation assay

Cells were plated in 96-well plates at 2×10^3 cells per well and cultured for 3 h. The medium was switched to medium conditioned by MSCs transfected with miR-143 or siNega. The cells were cultured for 0, 48, or 96 h. The absorbance at 450 nm was measured after incubation for 4 h with 10 μl of CCK8 solution (Cell Counting Kit-8; Dojindo, Kumamoto, Japan).

2.6. Migration assay

Conditioned medium, exosomes suspended in DMEM, or exosome-depleted conditioned medium derived from MSCs was added to 143B cells seeded at 1×10^5 cell/well in a 12-well plate, and incubated for 24 h. The 143B cells (6×10^3) suspended in 300 μl of serum-free DMEM were added to the 24-well upper chamber containing cell culture inserts with 8.0 μm pore polyethylene terephthalate track-etched membranes (BD Falcon Cell Culture inserts; BD Biosciences). The chambers were placed in 24-well plates and 500 μl of serum-free medium was added to the bottom wells of the multiwell insert assembly. After incubation for 8 h at 37 °C, the migrated cells were quantitated by counting the numbers of cell nuclei stained with NucBlue™ (Life Technologies) in three random fields on the membrane at a magnification of 100× under a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan).

2.7. Counting exosome particles

The sizes and numbers of the particles isolated from MSCs transfected with miR-143 or siNega were measured with the Izon qNano system using TRPS technology (Izon Science, Ltd.), as previously reported [18,19].

2.8. Immunoblotting analysis

Equal amounts of proteins from the cell extracts or the exosomes isolated from the equal amounts of conditioned medium were resolved on SDS–PAGE (Bio-Rad, Hercules, USA) and transferred to a PVDF membrane (Bio-Rad). The membrane was incubated with one of the following antibodies: anti-flotillin-1 (610820, 1:500; BD Biosciences), anti-CD81 (sc-7637, 1:200; Santa Cruz Biotechnology), anti-alix (sc-271975, 1:500; Santa Cruz Biotechnology), or anti-GAPDH (AB2302, 1:500; Millipore). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (sc-2005; Santa Cruz Biotechnology) or goat anti-rabbit IgG antibody (sc-2030; Santa Cruz Biotechnology) were used as second-antibody. The signal was detected with the chemiluminescence of Immuno-enhancer (Wako, Japan).

2.9. Statistical analysis

The results are presented as means ± SD or means ± SEM of four to six independent experiments. The data were analyzed with Steel or Steel-Dwass test ($P < 0.05$ was considered significant).

3. Results

3.1. Introduction of miR-143 into MSCs leads to increased extracellular miR-143

To determine whether the introduction of miRNAs into cells increases the extracellular miRNAs in the conditioned medium, real-time PCR was performed to measure the amounts of intra- and extracellular miR-143 after MSCs were transfected with miR-143. (The transfected cells were thoroughly washed with PBS before the medium was changed to remove any synthetic miR-143 or surplus liposome complex). Twenty-four hours after the transfection of miR-143, a dose-dependent increase in miR-143 accumulation was predictably observed in the MSCs (Fig. 1A). The accumulation of miR-143 observed in the conditioned medium was greater than that of the siNega control (Fig. 1B). These results show that the transfection of synthetic miR-143 into cells increases not only the level of intracellular miR-143 but also the level of extracellular miR-143.

3.2. Extracellular miR-143 can be transferred to osteosarcoma cells and inhibits their migration

To investigate whether extracellular miR-143 in the conditioned medium from MSCs can be transferred into 143B cells, we examined the expression of miR-143 in 143B cells after conditioned medium was added to them. The conditioned medium derived from MSCs transfected with miR-143 induced higher levels of miR-143 than any of the controls, including siNega, in a dose-dependent manner (Fig. 2A). Because the direct addition of 50 nM miR-143 without Lipofectamine (designated "naked miR-143" in Fig. 2A) did not change the levels of miR-143 in 143B cells, this effect was not attributable to the direct transfer of free miR-143 remaining in the cellular debris or conditioned medium. To investigate whether extracellular miR-143 in conditioned medium from MSCs can function in 143B cells, we performed cell proliferation and migration assays. miR-143 is known to be downregulated in osteosarcoma cells, and restoration of miR-143 inhibits cell proliferation and migration [10,11]. Conditioned medium including miR-143 did not significantly affect the proliferation of 143B cells (Fig. 2B). However, in the migration assay, conditioned medium containing miR-143 significantly and dose-dependently reduced the migration capacity of the cells compared with that of the control cells treated with MSC-conditioned medium or siNega (Fig. 2C and D). These findings are consistent with a previous report that extracellular miRNAs can be easily transferred to and function in recipient cells [16].

3.3. Extracellular miR-143 is mainly contained in exosomes

It has been reported that exosomes can shuttle between cells, and importantly, exosomes contain miRNAs [12]. To investigate the fraction containing miR-143 in conditioned medium, we isolated exosomes from medium conditioned by MSCs transfected with miR-143, using the ultracentrifugation method for exosome isolation (see Section 2) and quantified the levels of miR-143 in the exosome and exosome-depleted fractions of the conditioned medium. Higher levels of miR-143 were present in the exosome fraction extracted from medium conditioned by MSCs transfected with miR-143 than in the exosome-depleted conditioned medium, and the miR-143 in the exosomes accumulated in a dose-dependent manner (Fig. 3A). To investigate whether the secretion of exosomes from cells is influenced by the introduction of synthetic miR-143 into the cells, we examined the amount of exosomes released from miR-143-transfected MSCs. The numbers of nanoparticles, including exosomes, isolated from MSCs transfected with miR-143 were significantly higher than the numbers in nontransfected MSCs (Fig. 3B). The diameters of the nanoparticles were predominantly 60–180 nm, and the major peak in particle size was at 65–75 nm (data not shown). Exosome markers, such as CD81, flotillin-1, and alix, were increased by the introduction of miR-143 (Fig. 3C). Finally, we performed a cell migration assay to examine whether exosome-formed miR-143 can function in 143B cells as does the conditioned medium derived from MSCs transfected with miR-143. The exosomes and conditioned medium showed similar behaviors in suppressing cell migration. However, the cells treated with exosome-depleted conditioned medium did not show reduced cell migration (Fig. 3D and E). These results indicate that the miR-143 in the conditioned medium was mainly secreted as exosome-formed miR-143 and transferred to the recipient cells. Therefore, extracellular miR-143 derived from MSCs was able to reduce the migratory activity of 143B cells.

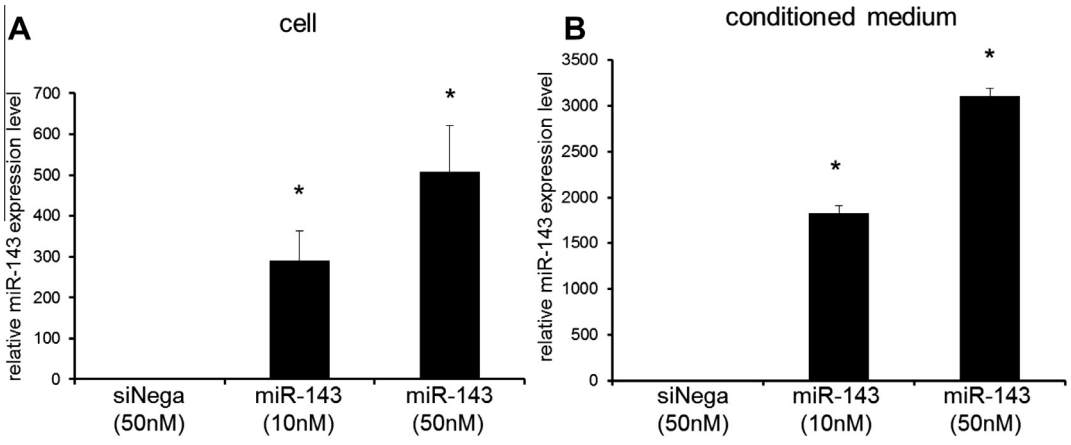

Fig. 1. miR-143 expression levels in MSCs and MSC-conditioned medium after transfection with miR-143. (A, B) Real-time PCR analysis of miR-143 expression in MSCs and MSC-conditioned medium after the cells were transfected with siNega (50 nM) or miR-143 (10 or 50 nM). RNU6 and cel-miR-39 were used as internal loading controls for cells and conditioned medium, respectively, to normalize the results. Data are the means ± SD. *$P < 0.05$ versus siNega.

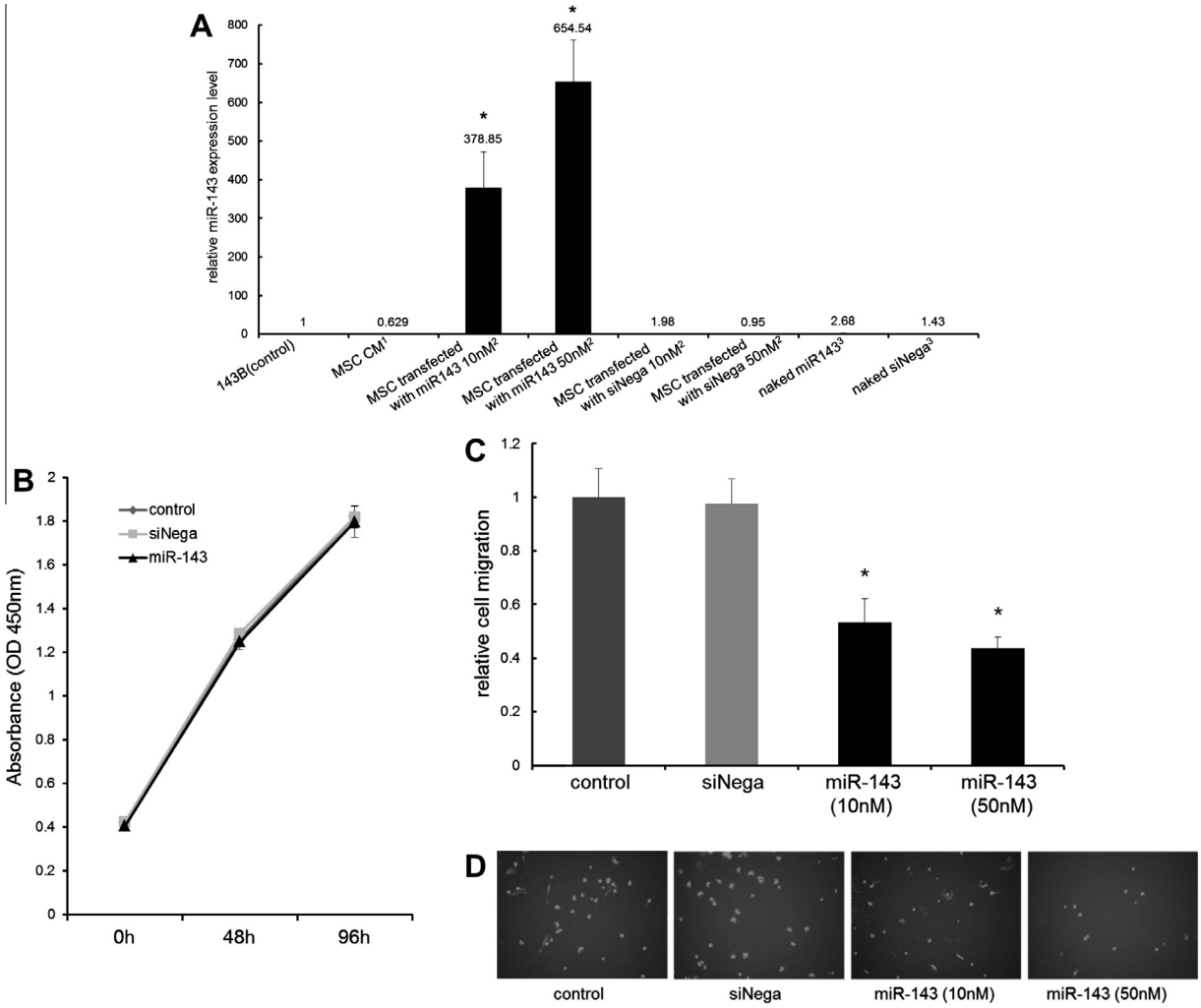

Fig. 2. Extracellular miR-143 from MCSs is transferred to 143B cells and inhibits their migration. (A) miR-143 levels in recipient 143B cells treated with MSC-conditioned medium (MSC-CM) including miR-143 or with naked miR-143 (control, 143B cells; 1, MSC-conditioned medium was added to 143B cells; 2, conditioned medium from MSCs transfected with miR-143 or siNega was added to 143B cells; 3, naked miR-143 [50 nM] or siNega [50 nM] was added to 143B cells. (B) Proliferation of three groups of 143B cells (control; 143B cells, siNega, miR-143; conditioned medium from MSCs transfected with siNega [50 nM] or miR-143 [50 nM]). (C) The migration of 143B cells treated with MSC-CM including miR-143 (or control, siNega [50 nM]) is shown as the relative cell migration 8 h after incubation. (D) Representative photographs of migrated 143B cells on the membrane at a magnification of 100×. Data are the means ± SD. $^{*}P < 0.05$ versus control. MSC-CM: conditioned medium from MSCs.

3.4. Exosomes deliver functional miR-143

To evaluate the efficiency of delivery of exosome-formed miR-143, we investigated the miR-143 expression in 143B cells transfected with miR-43 with the lipofection method or with exosomes. The transfection efficiency of exosome-formed miR-143 was less than that achieved with lipofection (Fig. 4A). However, the exosome treatment (50 nM) was less toxic (greater cell viability) to the 143B cells than lipofection (10 nM) (Fig. 4B). The transfection of siNega by the lipofection affect cell proliferation dose-dependently (data not shown). A migration assay was performed to examine whether exosome-formed miR-143 functioned as effectively as miR-143 delivered by lipofection. Although 143B cells transfected with miR-143 with the lipofection or with exosomes was significantly decreased migration activity compared with control (no treatment 143B cells), the inhibitory effects of miR-143 (50 nM) on migration activity were not significantly different in 143B cells delivered by lipofection or by exosomes (Fig. 4C and D). These results demonstrate that exosome-formed miRNAs are effectively transferred to recipient cells and function efficiently in the recipient cells.

4. Discussion

A therapeutic approach that has recently emerged as promising involves miRNA-based strategies using either miRNA mimics or antagonists, depending on the miRNA function affected and its status in the specific diseased tissue. However, despite its exciting potential, the delivery of miRNA-targeting agents must be resolved before their clinical application. A number of miRNA delivery systems have been developed [20], including liposomes [21] and peptide transduction domain–double-stranded RNA-binding domain (PTD–DRBD) [22]. However, their use is sometimes limited because synthetic materials are involved. In contrast, exosomes have potentially wide utility for the delivery of nucleic acids because they involve only biogenic substances and are readily transferred into target cells. It was recently reported that exosomes are effective carriers of siRNA and miRNA [17,23]. These studies showed that miRNA was introduced into exosomes after its transfection with electroporation or lipofection. Kosaka et al. showed that the secretion of miRNA was enhanced from cells transfected with an miRNA expression vector [16,24]. Our data demonstrate that the numbers of nanoparticles, including exosomes, isolated from do-

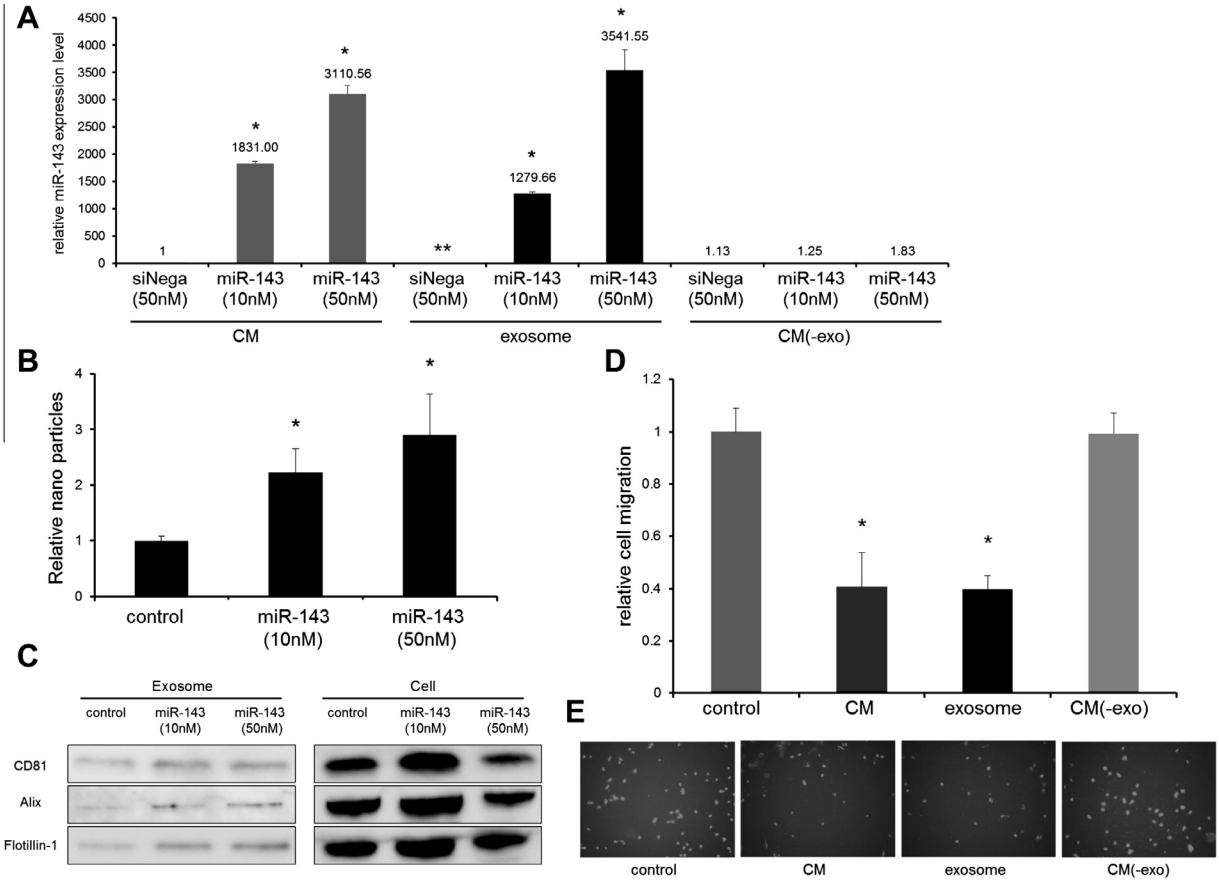

Fig. 3. Released extracellular miR-143 in exosomes. (A) Exosomes and exosome-depleted conditioned medium (CM(-exo)) were isolated from MSC-conditioned medium (CM) by ultracentrifugation. miR-143 expression in the CM, exosome, and CM(-exo) fractions was detected with real-time PCR. cel-miR-39 was used as the internal loading control. Data are the means ± SD. $^{*}P < 0.05$ versus siNega in CM. **Not determined. (B) Changes in the numbers of particles released after the transfection of MSCs with miR-143. $^{*}P < 0.05$ versus control. (C) Immunoblotting detection of exosome markers, flotillin-1, CD81, and alix proteins, in isolated exosomes from MSCs transfected with miR-143. (D) The migration capacity of each group of 143B cells (control, 143B cells; CM, conditioned medium from MSCs transfected with miR-143 [50 nM] was added to 143B cells; exosomes and CM(-exo), exosomes and CM(-exo) from MSCs transfected with miR-143 [50 nM] were added to 143B cells) is shown as the relative cell migration activity 8 h after incubation. Data are the means ± SD. $^{*}P < 0.05$ versus the control. (E) Representative photographs of migrated 143B cells on the membrane at a magnification of 100×. CM: conditioned medium from MSCs. CM(-exo): exosome-depleted conditioned medium from MSCs.

nor cells transfected with miRNAs increased in a dose-dependent manner. In other words, "exosome-formed miRNA", or exosomes that include the target miRNA, are easily obtained by introducing synthetic double-stranded miRNA into the donor cells.

The present study shows that the delivery efficiency of exosome-formed miR-143 was lower than that achieved with lipofection. However, the inhibitory effect on cell migration was similar in the migration assay. There are two possible explanations of why exosomes transferring less miR-143 than was transferred by lipofection suppressed cell migration equally well. One possibility is that although less miR-143 was transferred by the exosomes, it was sufficient to inhibit the migration of 143B cells. The other possibility is that miR-143 was incorporated with a protein complex, such as Ago2, in the exosomes [25] and was effectively transferred to the appropriate subcellular location to access its target mRNAs in the recipient cells. Our findings demonstrate that exosomes are transferred between cells, alter gene expression, and affect specific osteosarcoma cell functions, which means that exosomes function efficiently in the delivery of miRNAs.

Exosomes are secreted by cultures of various cell types, including cancer and immune cells, and are characteristic of the cells that produce them [15]. The physiological properties of exosomes remain incompletely understood. For instance, the contents of exosomes, including proteins, miRNAs, and mRNAs, vary according to the cell type that secretes them. Accordingly, exosomes derived from various cell types play different roles in biological phenomena [15]. In our study, MSCs were used as the cells to produce the exosome-formed miRNA because MSCs have a homing ability that allows them to migrate to sites of injury, inflammation, and tumors [26,27]. It has been suggested that MSCs secrete and transfer microvesicles containing specific miRNAs, which act in intercellular communication [28]. MSCs have been shown to deliver specific synthetic miRNA mimics into glioma cells and glioma stem cells, which inhibit their migration and self-renewal [29]. However, there are contradictory reports that exosomes derived from MSCs promote tumor growth [30]. Therefore, MSCs might be the appropriate cells in which to produce exosome-formed miRNAs. However, the appropriate choice of donor cells for exosome production might be important for the application of exosomes in the clinical context.

In summary, we have demonstrated that the introduction of synthetic miR-143 into cells increased the secretion of exosomes from those cells and the amount of exosome-formed miR-143 in the conditioned medium. Exosome-formed miR-143 was easily transferred into recipient cells and suppressed the migration of the 143B osteosarcoma cell line. Although the therapeutic application of exosome-formed miRNAs requires further *in vivo* studies, exosome-formed miRNAs have great potential for the targeted delivery of miRNAs in miRNA-supplementation therapies for various diseases, including cancers such as osteosarcoma.

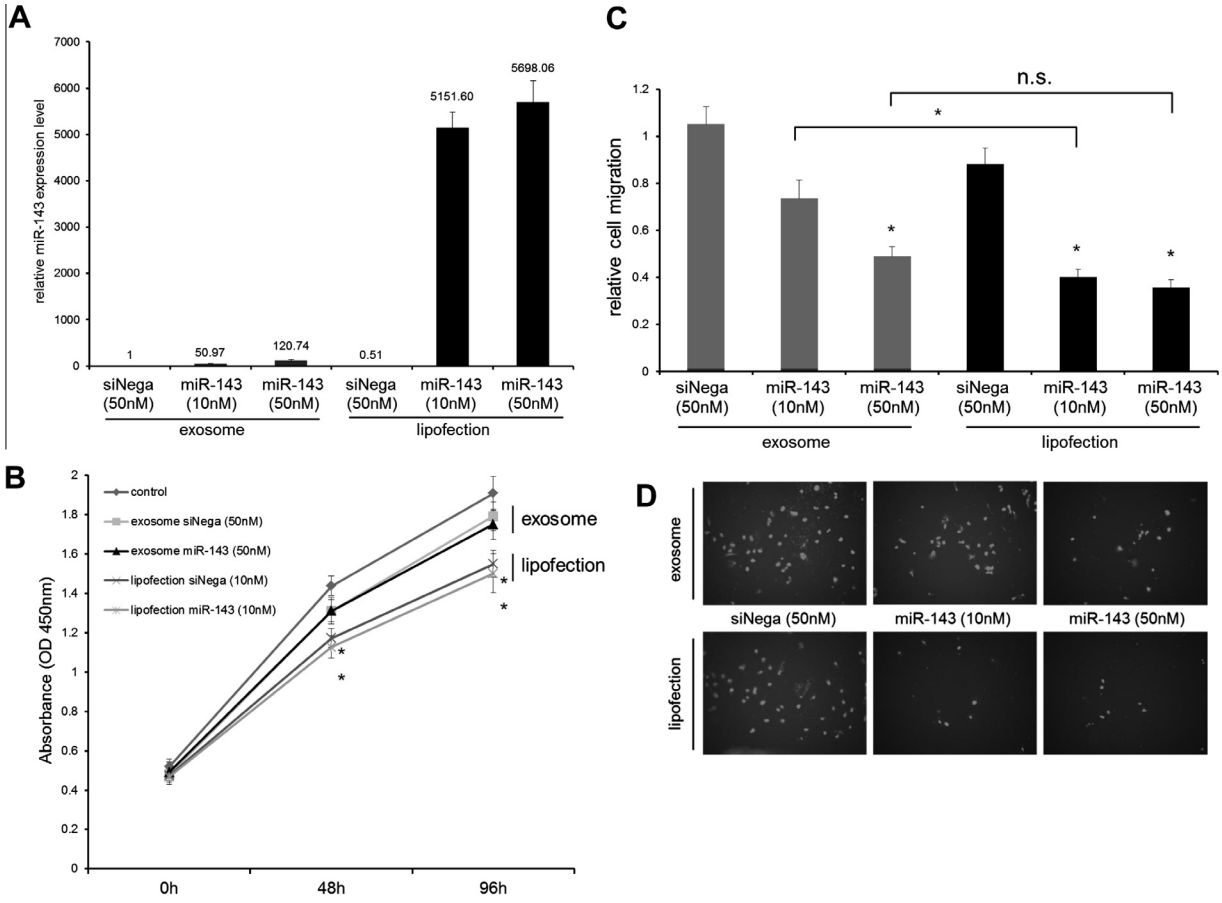

Fig. 4. Comparison of 143B cell transfection with exosome-formed miR-143 or lipofection. (A) Relative miR-143 expression levels in six groups of 143B cells (exosome group: exosomes of MSCs transfected with siNega [50 nM] or miR-143 [10 or 50 nM] were added to 143B cells; lipofection group: 143B cells transfected with siNega [50 nM] or miR-143 [10 or 50 nM]). (B) Proliferation in five groups of 143B cells (control: no treatment 143B cells; exosome: exosomes of MSCs transfected with siNega [10 nM] or miR-143 [50 nM] were added to 143B cells; lipofection: 143B cells were transfected with siNega [10 nM] or miR-143 [10 nM]). Data are the means ± SD. $^{*}P < 0.05$ versus control. (C) Relative cell migration in six groups of 143B cells with reference to untreated 143B cells. Data are the means ± SEM. $^{*}P < 0.05$ versus control (no treatment 143B cells), exosome versus lipofection. (D) Representative photographs of migrated 143B cells on the membrane at a magnification of 100×.

Acknowledgments

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