ANTICANCER RESEARCH *40*: 6319-6325 (2020)
doi:10.21873/anticanres.14652

Development of an Oncolytic Recombinant Vesicular Stomatitis Virus Encoding a Tumor-suppressor MicroRNA

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Abstract. *Background: Attempts have been made to enhance systemic therapy for osteosarcoma. In our previous study, the systemic administration of a vesicular stomatitis virus (VSV) improved the survival rates of mice with osteosarcoma but did not improve the long-term survival of the animals. Materials and Methods: In the present study, we developed a novel oncolytic VSV by incorporating tumor-suppressor microRNA143 (rVSV-miR143) to compare the antitumor effects of various doses (10×10^{-4}, 5×10^{-4}, and 1×10^{-4} multiplicity of infection) of rVSV-miR143 with those of VSV in vitro. Results: The cytotoxicity and migration-inhibitory effects of rVSV-miR143 on the osteosarcoma cells were significantly higher than those of VSV alone at a dose of 5×10^{-4} multiplicity of infection, indicating that rVSV-miRNA143 enhances the antitumor effect at certain doses. Conclusion: VSV incorporating tumor-suppressor miRNA143 demonstrated a synergistic antitumor effect on osteosarcoma cells in vitro.*

The standard treatment for osteosarcoma consists of surgery with adjuvant chemotherapy (1, 2). Especially when it is systemic, this treatment can suppress distant metastasis, improve the survival rate, and reduce the primary tumor, thus enabling the use of surgical procedures that help in conserving the function of the limb (1, 2).

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Key Words: Vesicular stomatitis virus, tumor-suppressor microRNA, osteosarcoma, systemic therapy.

Chemotherapy, which is the standard treatment for osteosarcoma, is sometimes ineffective because of the development of resistance to this treatment. Thus, new systemic therapies that enhance existing therapies are required. Various new chemotherapies for osteosarcoma are currently being studied (3, 4).

Oncolytic virus, which selectively replicates and proliferates in malignant tumor cells only, is expected to have a high tumoricidal effect on cells without the development of cross-resistance between conventional chemotherapy and radiation therapy.

In the present study, we used a vesicular stomatitis virus (VSV), which is an RNA virus that can be easily engineered to develop a new generation of VSV vectors. Our previous studies have shown that VSV has an oncolytic effect on osteosarcoma (5, 6) because it is sensitive to the interferon response and is tumor-specific owing to the deficiency of antiviral interferon signaling pathways in tumor cells (7-9). A new virus delivery system, the isolated regional perfusion method, developed for malignant bone and soft-tissue tumors demonstrated excellent targeting abilities in primary tumors (3). However, it has been pointed out that the established immunity against VSV antigens may compromise the antitumor effect of the intravenous VSV injection (6). In our previous study, systemic administration of VSV-Katushka in a murine model of osteosarcoma improved the survival of the animals but did not enhance their long-term survival (6). Katushka is a fluorescent protein with low cytotoxicity (10). A modification of the protein or the addition of a tumor-specific killing method might increase the efficacy of VSV in targeting tumor cells.

Studies have elucidated the biological effects and molecular biological mechanisms of cancer-related microRNAs (miRNAs) and identified several oncogenic and tumor-suppressor miRNAs (11-15). Among them, *miRNA-*

143 was found to be deeply involved in distant metastasis of osteosarcoma (13). VSV is an appropriate vector that can deliver miRNAs to tumor cells (16). The incorporation of miRNA target sites into oncolytic VSV results in a virus that is attenuated in normal cells and has reduced toxicity, while retaining the antitumor activity *in vivo* (17).

In this study, we focused on the strong ability of VSV to suppress tumor cell invasion and migration and aimed to develop a more potent oncolytic virus that combines the antitumor effects of both VSV and *miRNA143* by introducing the miRNA into the gene. The purpose of the study was to evaluate the oncolytic effect of the VSV-miRNA on osteosarcoma cells *in vitro*.

Materials and Methods

Cell lines and culture conditions. Highly metastatic mouse osteosarcoma cells (LM8) were obtained from the Cell Engineering Division of Riken BioResource Center (Tsukuba, Ibaraki, Japan). LM8 is a variant of a murine osteosarcoma cell line established by Asai *et al*. (18), in accordance with Fidler's method (19). Human osteosarcoma cells (143B, HOS, Saos-2, and MG-63) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Wako) in a fully humidified incubator at 37˚C and an atmosphere of 5% CO_2. Bone marrow-derived mesenchymal stem cells (BMSCs) were obtained from a 56-year-old male during hip surgery and cultured in Mesenchymal Stem Cell Basal Medium (Lonza Japan, Ltd., Chiba, Japan). The isolation and *in vitro* expansion of the BMSCs were performed as described previously (20). BHK-21 cells (ATCC; CCL-10) used for the construction and titration of VSV were maintained in DMEM with 10% fetal bovine serum (Gibco).

Virus generation. Recombinant VSV-*miRNA143* was generated using an established reverse genetics method (21, 22). Briefly, the *miR143* precursor gene (GenBank/DDBJ/EMBL accession number NR_105060, 949 bp) was synthesized by Eurofins Genomics (Tokyo, Japan) and inserted into the *Xho*I and *Nhe*I restriction sites of the plasmid pVSV-XN2, which contains the entire VSV genome sequence (Indiana serotype, GenBank accession number NC_001560) flanked by the T7 promoter and the hepatitis delta ribozyme plus the T7 terminator. The *Xho*I and *Nhe*I restriction sites were located between the G and L genes and flanked by the VSV transcription start and stop signals for the insertion of an additional gene (23). The resultant plasmid was transfected into BHK-21 cells constitutively expressing T7 RNA polymerase and the helper plasmids pIRES-N, pIRES-P, and pIRES-L to establish a newly recombinant virus, VSV-*miR143* (6, 24) (Figure 1). The viral titers of the stock solution [median tissue culture infective dose ($TCID_{50}$)/ml] were determined using the standard $TCID_{50}$ assay in BHK-21 cells (6).

In vitro miR143 expression in osteosarcoma and normal cells. Total cellular RNA was extracted from the osteosarcoma (LM8, 143B, HOS, Saos-2, and MG-63) and normal (MSC) cells using TRIzol Reagent (Life Technologies, NY, USA). The extracellular miRNAs were isolated using the mirVana miRNA Isolation Kit (Life Technologies), and the levels were determined in fixed volumes (1,000 μl) of conditioned media to which 5 μl of synthetic cel-miR-39 was added. The *miR-143* was amplified *via* real-time polymerase chain reaction (RT-PCR) using the TaqMan MicroRNA Assay (Life Technologies). Cel-miR-39 or *RNU6B* was used as the invariant control for the conditioned medium or cells, respectively. The ΔΔCt method was used to analyze the RT-PCR data. Three independent experiments were performed.

In vitro rVSV-miR143 infection assays (cytotoxicity assays). LM8 or MSC cells (3×10^5 cells/well) were incubated overnight in six-well plates and infected with rVSV-*miR143* at 1×10^{-2} multiplicity of infection (MOI). Forty-eight hours after infection, the cells were evaluated for cytopathic effects *via* bright-field microscopy.

In a separate experiment, BMSCs or fibroblasts (HDF3; Lonza) were seeded into 96-well plates at 4×10^3 cells/well overnight (volume, 100 μl) and infected with rVSV-*miR143* or VSV at 1×10^{-2} MOI. Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) based on WST-8 was used to measure the cell viability at designated time points after infection according to the manufacturer's recommendations. The absorbance was measured at 450 nm using a Model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA), and the cell viability was expressed as the percentage of viable cells compared to those of mock-infected control cells. Three independent experiments were performed.

In a separate experiment, LM8 cells seeded onto 96-well plates at 4×10^3 cells/well overnight (100 μl) were assigned at random to one of the three different dose groups (n=3 per group) as follows: 10×10^{-4}, 5×10^{-4}, and 1×10^{-4} MOI. The LM8 cells were seeded into 96-well plates at 4×10^3 cells/well (volume, 100 μl) and incubated overnight. Subsequently, they were infected with the different doses of rVSV-*miR143* or VSV. Cell Counting Kit-8 (Dojindo) based on WST-8 was used to measure the cell viability at designated time points after infection. The absorbance was measured at 450 nm, and the cell viability was expressed as the percentage of viable cells compared to the mock-infected control cells. Three independent experiments were performed.

In vitro rVSV-miR143 migration assays. Conditioned medium containing LM8 cells seeded at 4×10^3 cells/well in a 12-well plate was assigned at random to one of the three different dose groups (n=3 per group) as follows: 10×10^{-4}, 5×10^{-4}, and 1×10^{-4} MOI. Conditioned medium, rVSV-*miR143*, or VSV at each dose was added to LM8 cells (4×10^3 cells/well) in a 12-well plate and incubated for 24 h. The cells (6×10^3) were then suspended in 300 μl of serum-free DMEM and added to the upper chambers of a 24-well plate containing cell culture inserts with 8.0 μm pore polyethylene terephthalate track-etched membranes (BD Falcon cell culture inserts; BD Biosciences, Franklin Lakes, NJ, USA). The chambers were placed in the 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 quantified by counting the number of nuclei stained with NucBlue™ (Life Technologies) in three random fields at a magnification of 100× using a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan).

Statistical analysis. The results of the *miR143* expression levels in the osteosarcoma cells and BMSCs, absorbance in the cytotoxicity assay, and cell counts in the migration assay were compared using one-way analysis of variance or Student's *t*-test. All analyses were

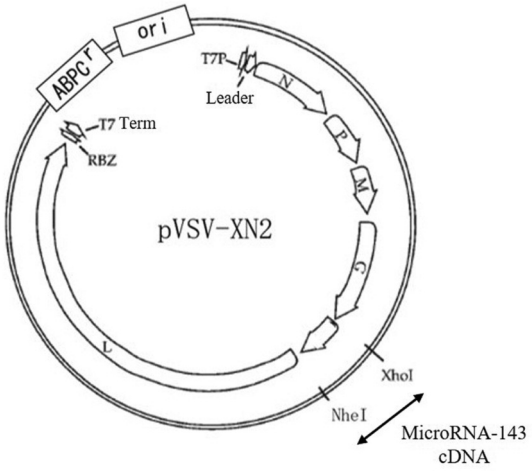

Figure 1. *Schematic drawing of pVSV-XN2-microRNA143. The vesicular stomatitis virus (VSV) Indiana genomic DNA consisting of the leader and trailer sequences and the N, P, M, G, and L genes flanked by the T7 promoter and T7 terminator. miRNA143 cDNA was inserted between the G and L genes using the XhoI and NheI restriction sites.*

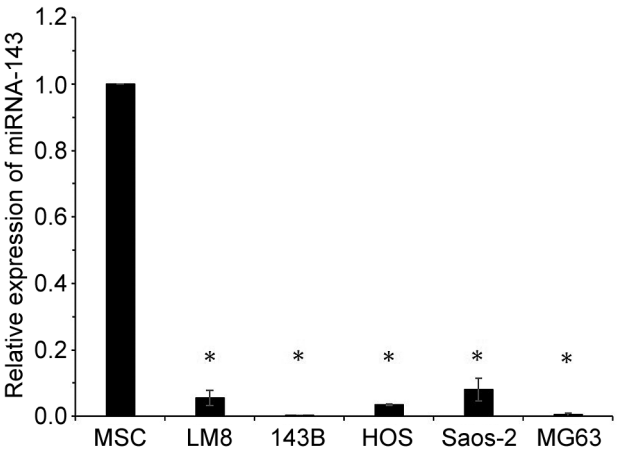

Figure 2. *MicroRNA143 expression in osteosarcoma cells and mesenchymal stem cells (MSCs) in vitro. miR-143 in mouse (LM8, 143B, and HOS) and human (Saos-2 and MG-63) osteosarcoma cells and normal MSCs were amplified by real-time polymerase chain reaction. miRNA-143 expression levels were suppressed in the osteosarcoma cells when compared to those in the MSCs. Data are presented as the mean±standard deviation (SD) obtained from six mice per group. *Significantly different from MSCs at p<0.05.*

performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corporation, Armonk, NY, USA). Values are presented as the mean±standard deviation (SD). A probability value of $p<0.05$ was considered statistically significant.

Results

miR143 expression levels in osteosarcoma and normal cells. The human and mouse osteosarcoma cell lines presented significantly lower levels of *miR143* expression than that in the normal MSCs (Figure 2). The expression of *miRNA-143* was suppressed in osteosarcoma cells compared to MSCs.

In vitro rVSV-miR143 infection assays (cytotoxicity assays). Furthermore, we examined whether there was a difference in the tumor cell-killing ability between the different doses of the virus. The osteosarcoma cells showed typical cytopathic effects by detaching themselves from the plate 48 h after being infected with rVSV-*miR143* at an MOI of 1×10^{-2}, whereas the morphology of the normal bone marrow stromal cells infected with the virus remained unchanged (Figure 3A). Moreover, the antitumor effect was not observed when normal cells were infected with either rVSV-*miR143* or VSV (Figure 3). Alternatively, in the WST-8 assay using LM8 cells, both rVSV-*miR143* and VSV demonstrated cytotoxic effects when infected at 10×10^{-4} MOI and no antitumor effects at 1×10^{-4} MOI (Figure 3C). At a dose of 5×10^{-4} MOI, the antitumor effect was observed only when rVSV-*miR143* was used (Figure 3C).

In vitro rVSV-miR143 migration assays. LM8 cells infected with rVSV-*miR143* (5×10^{-4} MOI) presented reduced migration when compared to those infected with VSV alone (Figure 4A and B). However, no differences in migration ability were noted between cells infected with rVSV-*miR143* and VSV at both 10×10^{-4} and 1×10^{-4} MOI (Figure 4B). These data support the results of the rVSV-*miR143* infection assays.

Discussion

In our previous study, we showed that the arterial injection of VSV into rats with osteosarcoma resulted in the inhibition of tumor growth and the targeting of the tumor by the virus (5). The intermittent intravenous injection of VSV-Katushka into mice with osteosarcoma suppressed lung metastasis and prolonged the survival of the animals (6). Some studies have demonstrated that VSV is specific to sarcoma cells (9, 25). Furthermore, Ryan *et al*. stated that VSV is a suitable vector that can deliver miRNAs to a wide range of tissues (16). Robert *et al*. stated that the incorporation of miRNA target sites into oncolytic VSV results in a virus that is attenuated in normal cells, with reduced *in vivo* toxicity, while retaining its antitumor activity (17). Hence, VSV is thought to be highly suitable for use as a vector for a tumor-suppressor miRNA that targets osteosarcoma cells.

The survival of a patient with a malignant tumor depends largely on the effectiveness of the systemic therapy. Therefore, developing stronger systemic therapies can lead to improved survival. Our previous study showed that systemic therapy

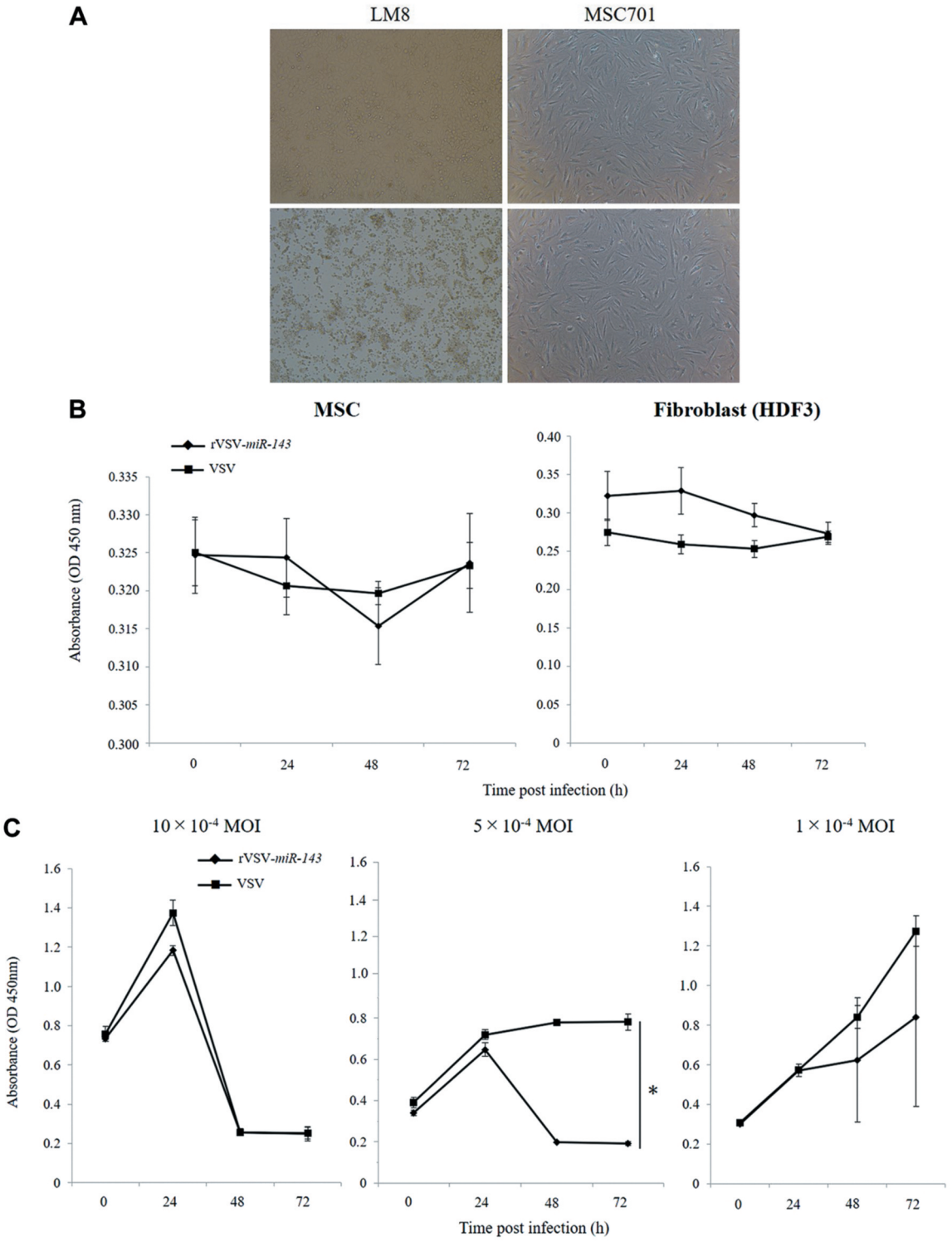

Figure 3. *A: Oncolytic vesicular stomatitis virus incorporating tumor-suppressor microRNA143 (rVSV-miR143) induced cytopathic effects in vitro. Mouse osteosarcoma cell lines (LM8) as well as normal bone marrow stromal cells (MSC701) at 48 h after infection with rVSV-miR143 [MOI 0 (upper panel) and 0.01 (lower panel)]. Mock-infected control cells are shown for comparison. B: Normal cell viability after infection of the cells with each of the viruses. Mesenchymal stem cells (MSCs)and fibroblasts (HDF3) were infected with VSV or rVSV-miR143. The absorbance was determined using the WST-8 assay and evaluated at the indicated times. C: The viability of the osteosarcoma cells (LM8) after infection with VSV or rVSV-miR143. The vertical axis indicates the average amount of absorbance obtained in the WST-8 assay. The absorbance was evaluated at the indicated times. Data are presented as the mean±SD obtained from three independent experiments. *Significantly different at $p<0.05$.*

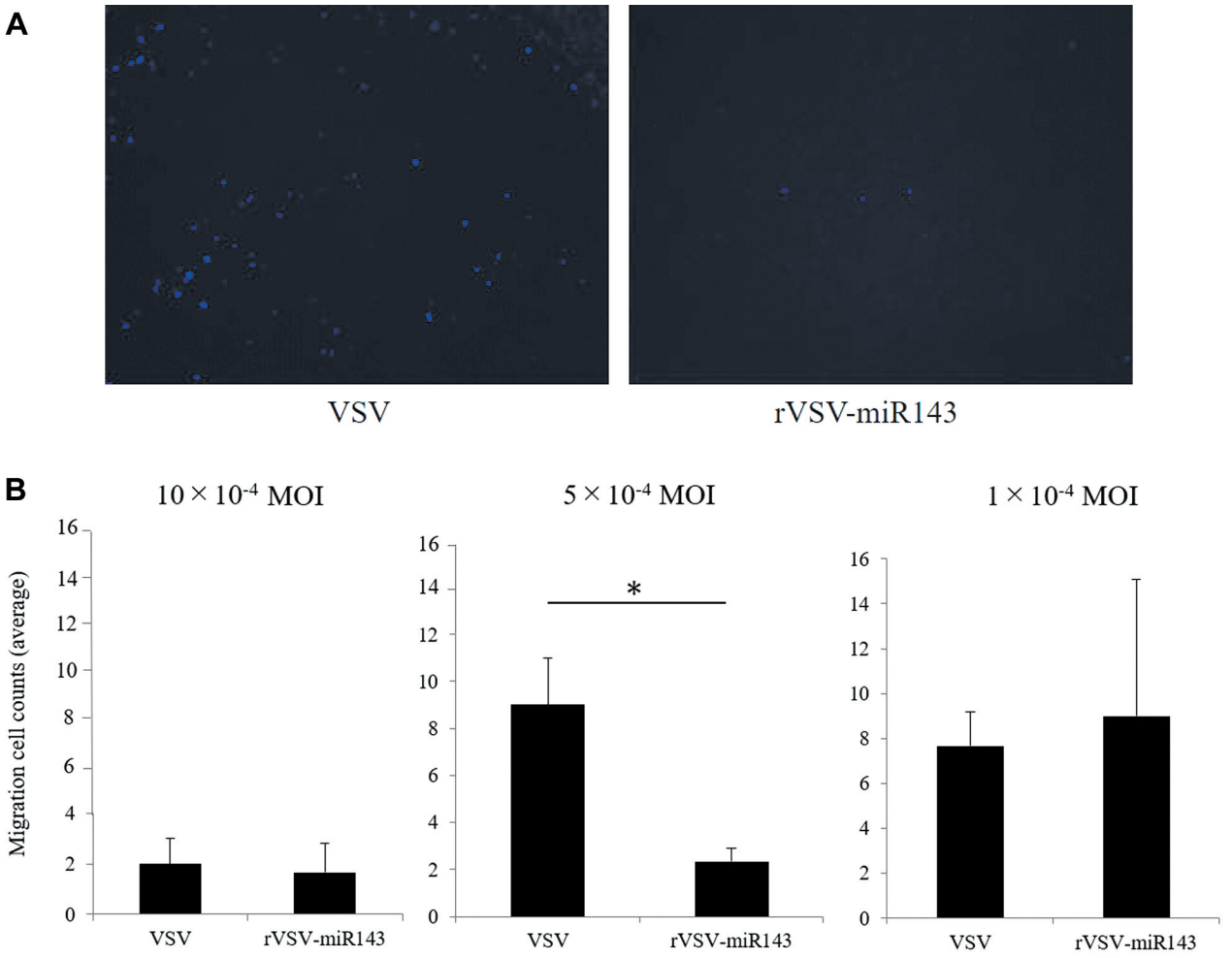

Figure 4. *A: Representative photographs of migrated LM8 cells on the membrane in the migration assay at a magnification of 100×. B: The cell counts of LM8 cells infected by each virus after 8 h of incubation are shown. Data are presented as the mean±SD obtained from three independent experiments. *Significantly different at p<0.05.*

with VSV improves survival (6). Other studies have demonstrated that *miRNA143* regulates metastasis in osteosarcoma (13, 14). On the basis of these findings, we presumed that a stronger antitumor effect might be obtained by combining these two factors.

To the best of our knowledge, there is no study on the synergistic antitumor effect of VSV and a tumor-suppressor miRNA. In the present study, a weak synergistic effect was noted. Although VSV incorporated with *miRNA143* enhanced the antitumor effect at some doses, the range of the doses was narrow. Some studies stated that the antitumor effect of VSV is very potent (24). One study reported that the time from infection to cytolysis is particularly rapid for VSV, with the viral load increasing by approximately 1,000-fold within 24 h (26). Therefore, VSV might have a more prominent impact on the killing of osteosarcoma cells than *miR143*.

The synergistic effect of tumor-suppressor miRNA integration into the osteosarcoma cells was not as expected in the current study. However, the effect was enhanced, and additional studies using new approaches for the incorporation of multiple tumor-suppressor miRNAs into VSV are required to determine the effects.

Most of the oncolytic virus studies used a DNA virus, such as adenovirus (27) or herpes simplex virus (28), which have to be genetically engineered to make it easier to infect tumor cells for the treatment of malignant tumors. The VSV used in this study is an RNA virus, which is easily engineered to develop new generations of VSV vectors. Besides VSV, other tumor-specific RNA viruses include reovirus (29), measles virus (30), poliovirus (31), and vaccinia virus (32). Among them, all except VSV have the potential to cause severe symptoms in humans, whereas VSV rarely infects humans and

manifests as mild flu-like symptoms (33). VSV is considered a safe oncovirus for humans and is expected to be used more often in the future. Furthermore, the antitumor effects of both VSV and *miR143* have been demonstrated in various carcinomas other than osteosarcoma (7, 8 34). The current study provides evidence that our technique can be applied to other types of cancer, including osteosarcoma, in the future.

In conclusion, VSV incorporating tumor-suppressor *miRNA143* demonstrated antitumor effects *in vitro*. Thus, the combination of VSV and tumor-suppressor miRNA might be used to produce a synergistic antitumor effect for the treatment of various cancer types.

Conflicts of Interest

All Authors declare that they have no competing interests.

Authors' Contributions

Study concepts and design: Tomohiko Sakuda, Tadahiko Kubo, and Nobuo Adachi. Data acquisition, analysis, and interpretation: Tomohiko Sakuda, Muhammad Phetrus Johan, Taisuke Furuta, and Takemasa Sakaguchi. Drafting and proofreading of the article: Tomohiko Sakuda, Tadahiko Kubo, Muhammad Phetrus Johan, Taisuke Furuta, Takemasa Sakaguchi, and Nobuo Adachi. Final approval of the article for publication: Tomohiko Sakuda, Tadahiko Kubo, Muhammad Phetrus Johan, Taisuke Furuta, Takemasa Sakaguchi, and Nobuo Adachi.

All Authors made a significant contribution to this article, approved the final version, and agreed with this submission to Anticancer Research.

Acknowledgements

This study was supported by a grant from the Japan Society for the Promotion of Science (grant no. 17K10970). The funding organization played no role in the study design, data collection/analysis, decision to publish, or preparation of the article.

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Received September 18, 2020
Revised September 30, 2020
Accepted October 2, 2020