Supporting Information

Exosome-mediated microRNA-497 delivery for anti-cancer therapy in a microfluidic 3D lung cancer model

Kyeongsoo Jeong,[a,†] Yeong Jun Yu,[b, †] Jae Young You,[a] Won Jong Rhee,[a, c,*] Jeong Ah Kim[b, d,*]

[a]*Department of Bioengineering and Nano-Bioengineering, Incheon National University, Incheon 22012, Republic of Korea*

[b]*Research Center for Bioconvergence Analysis, Korea Basic Science Institute, Chungbuk 28119, Republic of Korea*

[c]*Division of Bioengineering, Incheon National University, Incheon 22012, Republic of Korea*

[d]*Department of Bio-Analytical Science, University of Science and Technology, Daejeon 34113, Republic of Korea*

***To whom correspondence should be addressed.**

*W. J. Rhee, Tel: +82 32 835 8299; E-mail address: wjrhee@inu.ac.kr.

*J. A. Kim, Tel: +82 43 240 5068; Fax: +82 43 240 5158; Email: jakim98@kbsi.re.kr.

[†]These authors contributed equally to this work.

EXPERIMENTAL SECTION

miRNA synthesis

The miRNA-497 (miR-497) and miRNA-negative control (miR-NC) used in this study were purchased from Sigma-Aldrich in the USA. The sequence of miR-NC from *Caenorhabditis elegans* was used without the human gene sequence-related homology.

Cell culture

Human embryonic kidney 293T (HEK293T) cell was gifted from Prof. Kwon at Yonsei University in the Republic of Korea and human non-small cell lung cancer cell line (A549) was purchased from Korean Cell Line Bank (KCLB, Republic of Korea). They were cultured in Dulbecco's Modified Eagles Medium (DMEM, Corning, USA) with 10% (v/v) heat-inactivated FBS (Gibco, USA) and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific, USA). Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (C2517A, Lonza, USA) and cultured in the supplemented endothelial cell growth medium (EGM-2, Lonza, USA).

Preparation of exosomes

For the production of exosome, HEK293T cells were cultured in medium supplemented with the exosome-free FBS that was produced according to our previous reports.[1,2] The exosomes were isolated using ExoQuick-TC™ (System Biosciences, USA) according to the manufacturer's instructions. For isolation of the exosomes by ultracentrifugation (UC), a differential centrifugation method was used according to our previous report.[2]

Transfection of exosome with miRNAs

To generate miR-NC- and miR-497-loaded exosomes (hereafter referred as miR-NC exosome and miR-497 exosome), the isolated exosomes were transfected with miR-497 or miR-NC using a Lipofectamine RNAiMAX Reagent kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Briefly, 200 pmol of miRNAs were mixed with the reagent and incubated with 1×10^{11} exosomes for 6 h at 37 ºC. Then, the mixture was ultra-filtered with a 100-kDa filter (Merck Millipore, Ireland) to remove free miRNAs unloaded in the exosomes.

Nanoparticle tracking analysis (NTA) and zeta-potential analysis

The concentration and size distributions of the exosomes were analyzed using a NanoSight NS300 (Malvern, UK) equipped with NTA software (Version 3.2, Malvern, UK). The zeta potential of the exosomes was analyzed using Zetasizer Nano ZS (Malvern, UK) equipped with Zetasizer software (Version 7.12, Malvern, UK).

Transmission electron microscope (TEM)

For examination by the TEM, the exosomes were absorbed onto a carbon-coated grid, and they were stained negatively with 2% uranyl acetate for 1 min. Then, the samples were dried further for 15 min and observed using a JEM-1400plus electron microscope (JEOL, Japan) at the Korea Basic Science Institute in the Republic of Korea.

Cell proliferation and cytotoxicity assays

To assess cell proliferation, a WST-1 assay was performed using an EZ-Cytox kit (DoGenBio,

Republic of Korea) according to the manufacturer's instructions. To evaluate the cytotoxicity of the exosomes, cell viability was assessed using a trypan blue exclusion assay. Cells were seeded on a 96-well plate at 5×10^3 cells cm^{-2}, and the exosomes were treated in each well. The proliferation and viability of the cells were analyzed, respectively, at 0, 24, 48, and 72 h after the exosomes were treated.

Exosomal miRNA stability assay

To measure the stability of the miRNA-loaded in exosomes, the free miR-497s, the free miR-497s mixed with exosomes, and miR-497 exosomes were added to the medium, which was incubated at 37 ºC. MiRNAs were extracted with a miRNeasy mini kit (Qiagen, Germany), and cDNA was synthesized using a miScript RT II kit (Qiagen, Germany) according to the manufacturer's instructions. The miR-497 level was examined by a quantitative real-time PCR (qRT-PCR) using a miScript SYBR green PCR kit (Qiagen, Germany) according to the manufacturer's instructions.

Western blot analysis

Cell pellets and the extracted exosomes were dissolved in lysis buffer (RIPA, Elpis Biotech, Republic of Korea) that contained a protease inhibitor cocktail (Thermo Scientific, USA). The concentration of the lysed protein was determined using a BCA protein assay kit (Thermo Scientific, USA) according to the manufacturer's instructions. 30 μg of protein lysates of each sample were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, USA). After blocking, the membranes were incubated overnight at 4 ºC using primary antibodies

including anti-Alix, anti-TSG101, anti-CD81 (Abcam, UK) and anti-calnexin (Cell Signaling Technology, USA). After the membranes were washed, they were incubated with HRP-conjugated secondary antibodies (Abcam, UK) for 1 h at 37 ºC. Detection reagent (GE Healthcare, UK) was used for the chemiluminescence reaction, and the band images were obtained using the ChemiDocTM XRS+ System (Bio-Rad, USA). For the analysis of the intracellular protein, vascular endothelial growth factor-A (VEGF-A) from A549 cells and vascular endothelial growth factor receptor-2 (VEGFR-2) from HUVECs were measured with the method described above. Anti-VEGF-A (Abcam, UK) and anti-VEGFR-2 (Cell Signaling Technology, USA) antibodies were used as the primary antibodies, and the subsequent process was the same as described above.

Transwell migration assay

A migration assay of A549 cells was performed using a Transwell insert made of a polycarbonate membrane with pore sizes of 8.0 μm (Corning, USA). The A549 cells, which were exposed to miR-NC or miR-497 exosomes for 48 h on a 6-well plate, were transferred on the apical side of the Transwell insert at 5×10^4 cells per well in a serum-free medium, and a medium that contained serum was added to the receiver well for 24 h to induce migration through the pores of the membrane. After removing the cells that remained on the apical side, the cells that migrated to the basolateral side were fixed with 4% paraformaldehyde in PBS for 2 min at room temperature (RT). After rinsing with PBS, 0.1% crystal violet stain solution (Sigma-Aldrich, USA) was treated in cells for 10 min at RT. The stained cells were rinsed thoroughly with PBS and completely dried before observation with a light microscope. This experiment was repeated three times, and the five fields of views for each condition were counted and averaged.

Quantitative real-time RT-PCR (qRT-PCR)

The qRT-PCR was performed to identify the miR-497 level or the mRNA level of VEGF-A, YAP1, HDGF, CCNE1, E-cadherin, Snail and Vimentin. Total mRNAs were extracted from the cells using a Tri-RNA Reagent (Favorgen Biotech Corp., Taiwan), and they were quantified using a microplate reader (BioTek Instruments, USA). The cDNA was synthesized using a miScript RT II kit (Qiagen, Germany), and the miR-497 level was examined by qRT-PCR using a miScript SYBR green PCR kit (Qiagen, Germany). RNU6B and β-actin were used as internal controls for the miRNAs and mRNAs, respectively. The PCR was performed using a Thunderbird® SYBR® qPCR Mix with 40 cycles (TOYOBO, Japan) on a QuantStudio 3 Real-Time PCR machine (Thermo Fisher Scientific, USA). The sequence of primers is listed in Supplementary Table S1.

Microfluidic device fabrication

A microfluidic device for 3D cell cultures was designed using AutoCAD software (Autodesk, USA), and the device was fabricated using a standard soft lithography protocol.[3] The microfluidic chip has one gel channel in the center and two medium channels on the sides. The trapezoidal-shaped micropillars were arrayed along the gel channel with a certain gap distance (200 μm) between them; this is for filling the solution using surface tension. The widths of the gel channel and medium channel are 1.3 mm and 1 mm, respectively. The total length of the channels was about 15 mm and their depth was about 200 μm. After fabrication, the Sylgard 184 elastomer, poly-dimethylsiloxane (PDMS) (Dow Corning, USA) was poured on the Si master mold to a thickness of about 3 to 4 mm, and baked at 80 °C for 2 h for polymerization. After peeling off the PDMS replica, the inlet and outlet holes (diameter: 4 mm each) connected with gel and medium

channels were punched. Finally, the PDMS layer was bonded onto the cover glass (24 mm × 24 mm). Just after bonding, the surfaces of the channels were coated with 1 mg mL^{-1} of poly-D-lysinehydrobromide (PDL) (Sigma-Aldrich, USA) for 4 h to increase the electrostatic interactions between the gel and channel surfaces. The channel was washed thoroughly three times with D.W.[4,5] Then, the microfluidic devices were dried in an oven at 80 °C.

Tumor migration and angiogenesis assays in a microfluidic device

Type I collagen (Corning, USA) diluted to 2 mg mL^{-1} in PBS was used as a scaffold material. We used the recipe of the collagen solution that was described in previous reports.[4-6] The collagen solution was injected through the gel channel, and incubated for 30 min at 37 ºC for gelation. After gelation, a more diluted collagen solution (35 μg mL^{-1} in PBS) was introduced into the medium channel for coating to enhance the attachment of cells on the surfaces of the device. The device was placed in a humidified 5% CO_2 incubator for 30 min, and it was rinsed with medium. For the angiogenesis experiments, a suspension of cells (2×10^6 cells mL^{-1}, 40 μL) was loaded into one medium channel; the cells were instantly attracted to the sidewalls of the gel channel because of the flow induced by the hydraulic pressure across the gel. The cells attached to the gel sidewalls are expected to begin sprouting more easily by the VEGF gradient than the cells on the bottom surface. After 30 min, the two medium channels were washed and filled with the same volume of medium.[5] After seeding, the device was placed in an incubator at 37 ºC for 1 h to allow the cells to attach to the surface of the collagen scaffold. Then, a recombinant human VEGF-A (R&D Systems, USA), which was diluted in the medium to a final concentration of 100 ng mL^{-1}, was introduced into the medium channel on the opposite side to the HUVEC channel to generate a VEGF-A gradient via the collagen scaffold. MiR-497 exosomes or miR-NC exosomes were added to the HUVEC channel. The medium that contained VEGF-A, miR-497 exosome, or miR-NC

exosome was freshly replaced every day, and angiogenic sprouting was monitored for 4 days. For the migration of A549 cells, cells were seeded into one side of the channel at a concentration of 2×10^6 cells mL^{-1} after the gelation of collagen mentioned above. After the cells were attached, the miR-497 exosomes or miR-NC exosomes were added only to the A549 channel, and the migration of A549 cells toward the gel was monitored for 4 days.

Co-cultures of HUVECs and A549 cells in a microfluidic device

For the co-culture experiments, first, type I collagen (Corning, USA) was injected into the gel channel. After gelation of the collagen, cell channel was coated with the diluted collagen solution mentioned in the previous section. A suspension of A549 cells with a concentration of 2×10^6 cells mL^{-1} was introduced in the medium channel 1 day prior to the seeding of the HUVECs. A suspension of HUVECs with the concentration of 2×10^6 cells mL^{-1} was introduced into the medium channel as mentioned in angiogenesis assays, which was the opposite side of the A549 channel. The medium for co-culture was supplied with a 1:1 mixture of EGM-2MV and the supplemented DMEM. Beginning on the day of the seeding of the HUVECs, miR-497 exosomes were added to both of the cell channels, and the cells were co-cultured for an additional 4 days. The cell channels were replaced every day with medium that contained the miR-497 exosomes.

Image analysis

The images that were acquired were analyzed quantitatively by using an Image *J* software. In the experiments concerning the angiogenesis of HUVECs, 11-18 region of interest (ROI) images of each condition were taken, and all binary and thresholded images were analyzed. Likewise, for

the migration area of A549 cells toward the gel, the binary images were obtained from 15- 30 ROI images of each condition, and the proportion of the fluorescence pixels were calculated for every image. To assess the tube formation of HUVECs, the total number of tubes formed in HUVECs cultured in the channel were counted with three different devices, and the areas of tubes were measured using an Image *J*.

Immunostaining

To assess the angiogenic sprouting of HUVECs and the migration of A549 cells, the cells were fixed with 4% paraformaldehyde at RT for 20 min, and permeabilized with 0.1 % Triton X-100 for 20 min. Actin filaments and nuclei were stained with phalloidin (1:40, Sigma-Aldrich, USA) and Hoechst 33342 (1:1500, Thermo Fisher Scientific, USA), respectively. After co-culturing the HUVECs and A549 cells in a microfluidic device, the cells were fixed and permeabilized, this was followed by blocking with 5% (w/v) bovine serum albumin (BSA, MP Biomedicals, France) for 1 h at RT in DBPS containing 0.1% (v/v) Triton X-100. The anti-CD31 (1:100, Abcam, USA) and anti-EGFR (1:300, Abcam, USA) antibodies were used as the primary antibodies, and Alexa-fluor-488 and 594 conjugated antibodies (1:1000, Invitrogen, USA) were used as the secondary antibodies. The nuclei were stained with Hoechst 33342 (Thermo Fisher Scientific, USA). The cells were monitored, and fluorescence images were obtained using a fluorescence microscope (Axio Observer. Z1, Carl Zeiss, Germany).

Statistical analysis

All of the experiments were conducted with triplicate samples at least, and all numerical data were expressed as mean ± S.D. The significance of the difference between the two independent groups was determined using a two-tailed Student's *t*-test. Differences were considered statistically significant at $p < 0.05$. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

Table S1. List of primer sequence for qRT-PCR

Gene name	Forward primer sequences	Reverse primer sequences
VEGF-A	5’-ATCTTCAAGCCATCCTGTGTGC-3’	5’-GCTCACCGCCTCGGCTTGT-3’
YAP1	5’-GTAGCCAGTTACCAACACTG-3’	5’-CTGTTCAGGAAGTCATCTGG-3’
HDGF	5’-CTCAAATCCCCGACGTTTCCAG-3’	5’-GTTGGGCTTGCCAAACTTCTCC-3’
CCNE1	5’-GAACTGTGTCAAGTGGATGGTTCC-3’	5’-CCCGCTGCTCTGCTTCTTAC-3’
E-cadherin	5’-GCTGGACCGAGAGAGTTTCC-3’	5’-GGTGTATACAGCCTCCCACG-3’
ZO-1	5’-GCGGTCAGAGCCTTCTGATC-3’	5’-CATGCTTTACAGGAGTTGAGACAG-3’
Snail	5’-TTCTCTAGGCCCTGGCTGCTA-3’	5’-CTTGACATCTGAGTGGGTCTGGA-3’
Vimentin	5’-AATGCGTCTCTGGCACGTCTTG-3’	5’-CTTCTGCCTCCTGCAGGTTC-3’
β-actin	5’-’-AGCTGAGAGGGAAATTGTGCG-3’	5’-GCAACGGAACCGCTCATT-3’

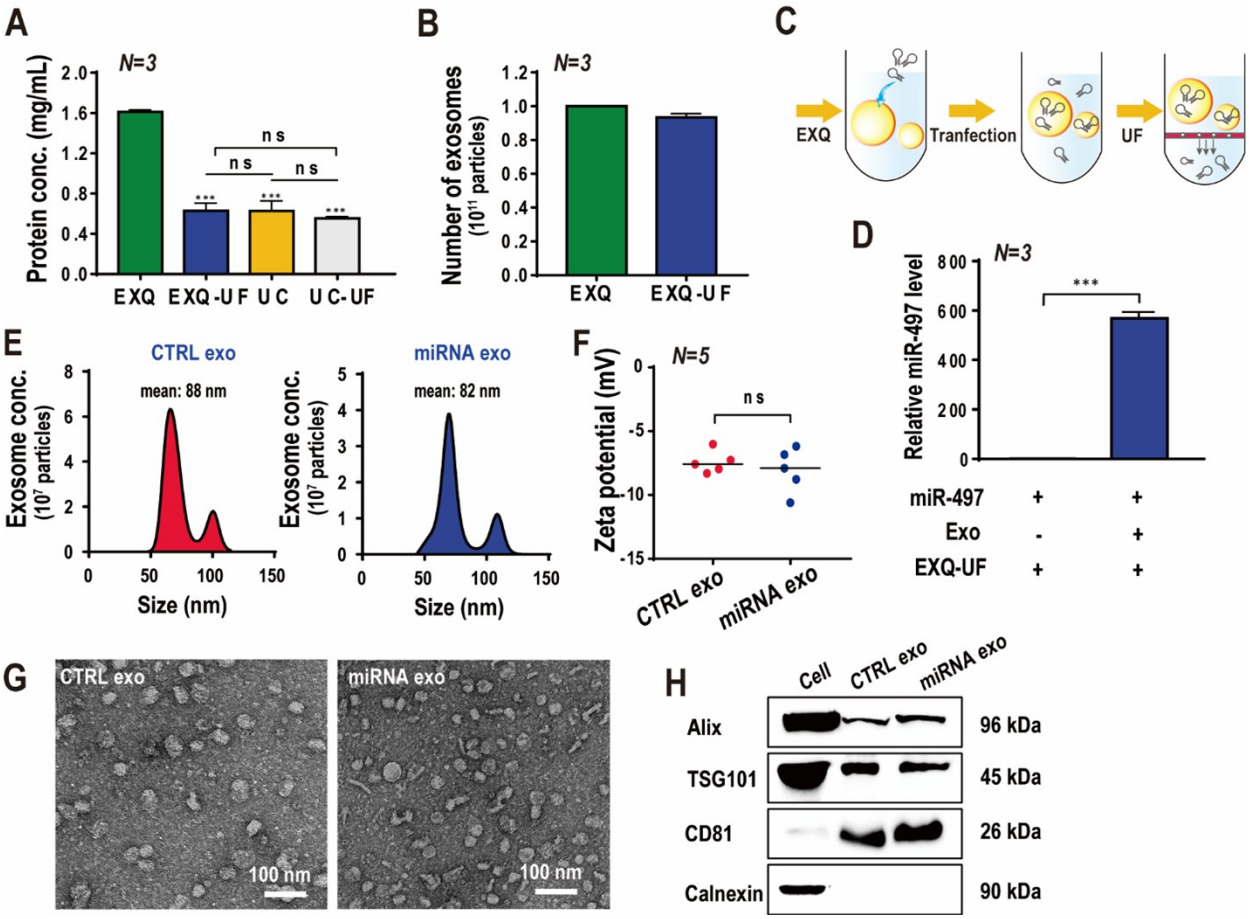

Figure S1. Isolation, purification and characterization of exosomes. (A) Quantification of protein contamination in HEK293T-derived exosomes after isolation using different isolation methods, i.e., EXQ: ExoQuick-TC™ method; UC: differential ultracentrifugation method; and UF: ultrafiltration. (B) Comparison of the total number of exosomes isolated using EXQ and EXQ-UF. (C) Optimized method for exosome isolation and purification. Exosomes were isolated using EXQ, transfected with miRNAs, and purified using UF to remove free miR-497s that were not loaded into exosomes. (D) Relative miR-497 level in the upper supernatant after UF with and without exosomes. (E) The size distribution of exosomes with and without miRNA loading analyzed by NTA. CTRL exosome represents the isolated exosome only using ExoQuick-TC™ before UF. (F) Zeta potential of exosomes with and without miRNA loading. (G) TEM images of exosomes with and without miRNA loading. (H) Western blot analysis with exosomes and cells.

Alix, TSG101, and CD81 are exosomal markers, and calnexin is a non-exosomal marker. All numerical data were expressed as mean ± S.D. (ns, not significant; ***, $p < 0.001$).

A

Amount of media required to obtain 10^{11} exosomes	
EXQ-UF	6.71 mL
UC-UF	73.8 mL

B

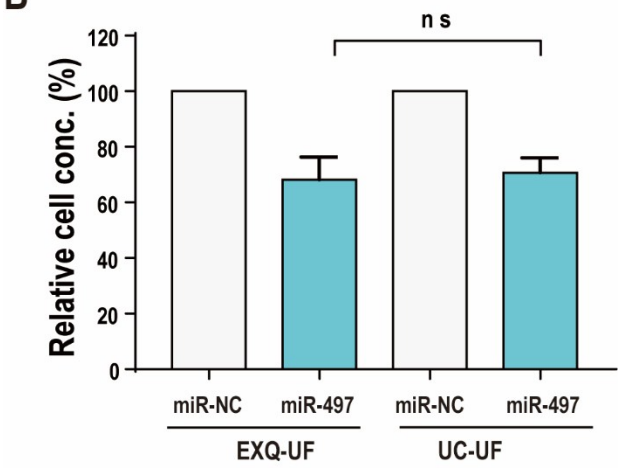

Figure S2. (A) Amount of medium required to obtain 10^{11} exosomes. Approximately 11 times higher volume of medium is required when isolating exosomes by UC as opposed to EXQ-UF to obtain the same number of exosomes. (B) Anti-proliferation effect of miRNA-loaded exosomes isolated by EXQ-UF and UC-UF. MiR-NC exosomes and miR-497 exosomes were treated in A549 cells, and the cell growth was measured using a WST-1 assay. The anti-proliferation effect of miR-497 exosomes isolated by EXQ-UF was very similar to that of exosomes isolated by UC-UF. (ns, $p>0.05$).

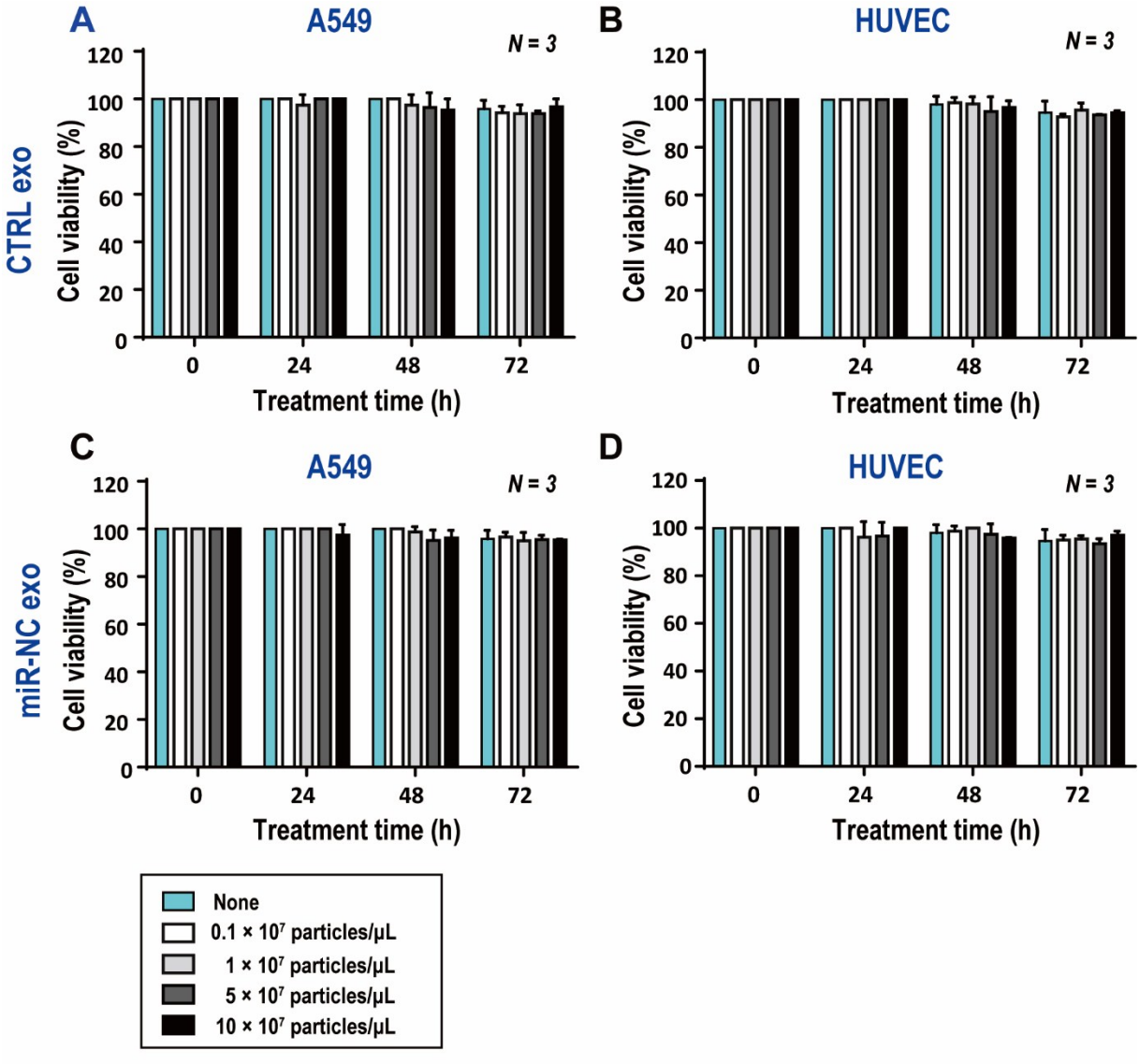

Figure S3. Dose- and time-dependent cytotoxicity of miRNA-loaded exosomes in A549 cells and HUVECs. A549 cells and HUVECs were seeded on a 96-well plate, and cell viability was measured using a trypan blue exclusion assay at 0, 24, 48, and 72 h after the treatment of the CTRL exosomes (A and B) and the miR-NC exosomes (C and D) ranging from 0 to 10^8 particles μL^{-1}. Three-independent experiments were performed. Cell viability was calculated as the percentage of live cells in the entire population at each condition. All of the data were not statistically significant when compared to the non-treated control cells.

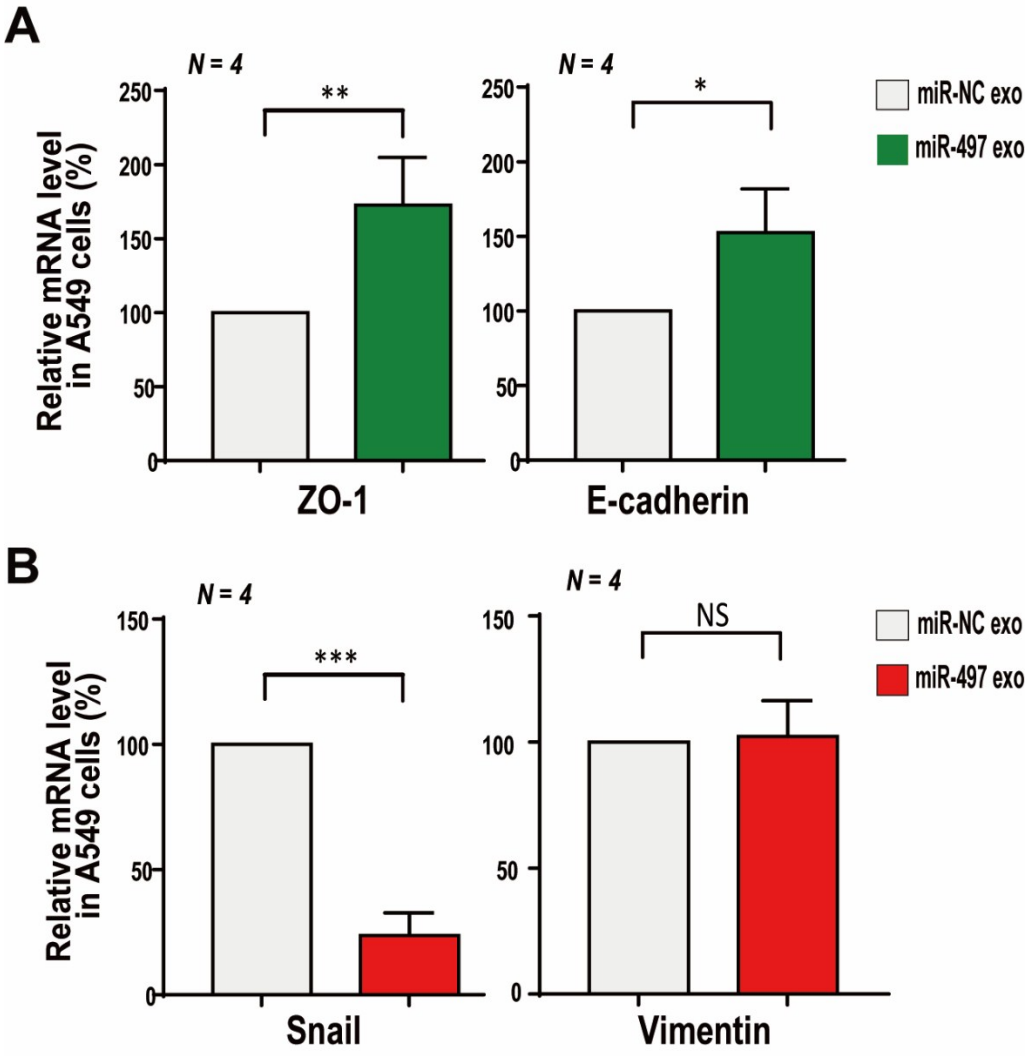

Figure S4. Differential expression level of EMT-related genes by miR-497 exosomes. Cells were pre-treated with miR-497 exosomes or miR-NC exosomes, and their mRNA level of EMT-related genes were evaluated using qRT-PCR. (A) epithelial markers, E-cadherin and ZO-1, and (B) mesenchymal markers, Snail and Vimentin. The effect of miR-497 exosomes was compared to the effect of miR-NC exosomes. All numerical data were expressed as mean ± S.D. (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

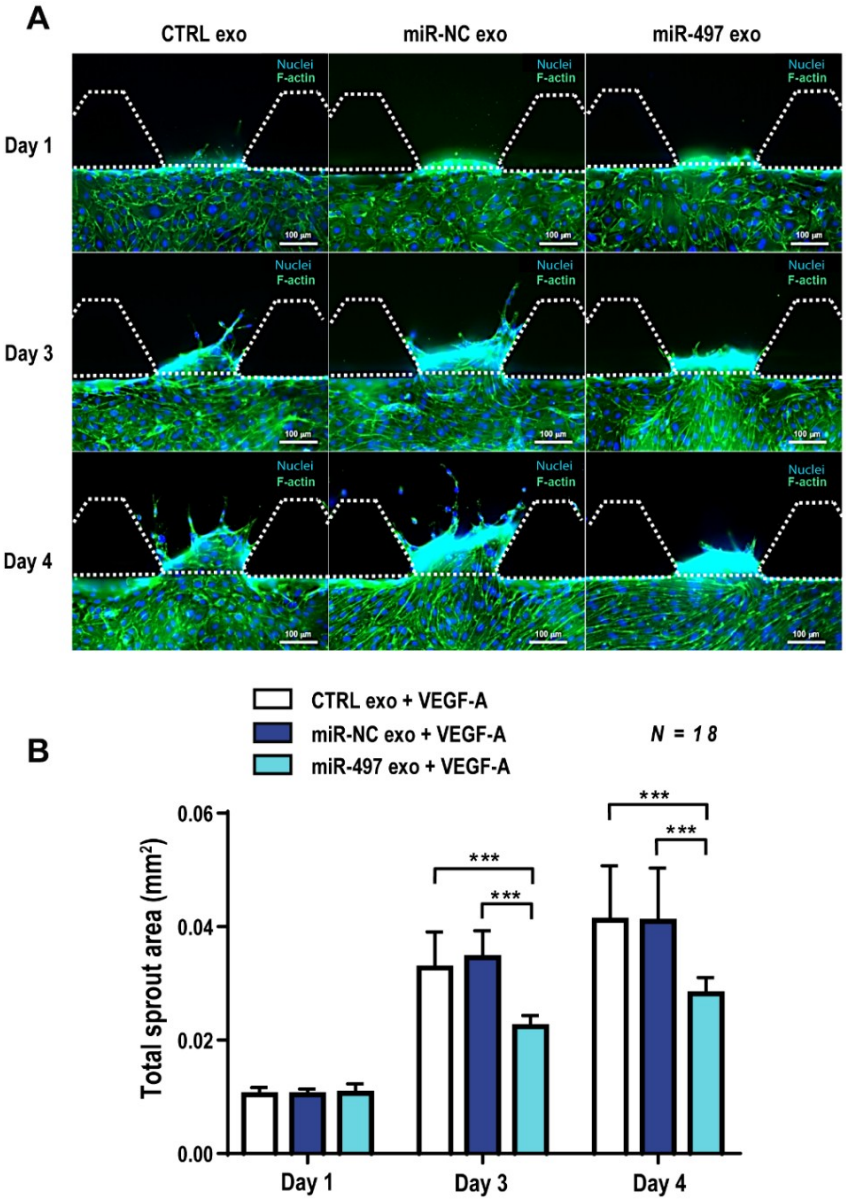

Figure S5. Time-dependent, anti-angiogenic effect of HUVECs cultured in a 3D microfluidic device. When the HUVECs were attached, VEGF-A was added to the opposite side channel across the gel channel to induce VEGF-mediated angiogenesis from HUVECs. The effect of miR-497 exosomes was compared to the effects of miR-NC exosomes and the control exosomes. A time-dependent angiogenic pattern of HUVECs was observed for 4 days, and the angiogenic sprout area was measured by *Image J* from the images that were obtained. All numerical data were expressed as mean ± S.D. (***, $p < 0.001$).

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