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Neutral Sphingomyelinase 2 (nSMase2)-dependent Exosomal Transfer of Angiogenic MicroRNAs Regulate Cancer Cell Metastasis*[S]

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Background: Contribution of exosomal microRNAs to cancer metastasis remains unknown.
Results: Exosomal angiogenic microRNAs secreted by metastatic cancer cells promote the metastasis through the activation of endothelial cells.
Conclusion: Horizontal transfer of exosomal miRNAs from cancer cells can dictate the microenviromental niche for the benefit of the cancer cell.
Significance: This is the first to connect cancer metastasis to the exosomal microRNA *in vivo*.

The release of humoral factors between cancer cells and the microenvironmental cells is critical for metastasis; however, the roles of secreted miRNAs in non-cell autonomous cancer progression against microenvironmental cells remain largely unknown. Here, we demonstrate that the neutral sphyngomyelinase 2 (nSMase2) regulates exosomal microRNA (miRNA) secretion and promotes angiogenesis within the tumor microenvironment as well as metastasis. We demonstrate a requirement for nSMase2-mediated cancer cell exosomal miRNAs in the regulation of metastasis through the induction of angiogenesis in inoculated tumors. In addition, miR-210, released by metastatic cancer cells, was shown to transport to endothelial cells and suppress the expression of specific target genes, which resulted in enhanced angiogenesis. These findings suggest that the horizontal transfer of exosomal miRNAs from cancer cells can dictate the microenviromental niche for the benefit of the cancer cell, like "on demand system" for cancer cells.

The secretion of humoral factors from cancer cells to microenvironmental cells is essential for metastasis during cancer development (1). Although microRNAs (miRNAs)[3] are known as tumor suppressors of cell autonomous malignancy phenotypes such as metastasis (2) and multidrug resistancy (3), the roles of miRNAs in non-cell autonomous cancer progression against microenvironmental cells remain largely unknown. The existence of secretory RNA has been known for many years (4, 5), and recent reports have shown that miRNAs (6), which regulate various types of biological phenomena through the regulation of a variety of target genes, are secreted from cells via the exosome (7, 8). These findings have raised the possibility that RNAs, including miRNAs, may serve as novel humoral factors in cell-cell communication (9). We recently demonstrated that miRNAs are released through neutral sphingomyelinase 2 (nSMase2)-regulated secretory machinery and that these secretory miRNAs are transferable and functional in recipient cells (10). Furthermore, we also found that a tumor-suppressive miRNA secreted from non-cancerous cells via this pathway could be transported between cells and exert gene silencing in the recipient cancer cells, thereby leading to an inhibition of cancer cell growth (11). In the last few years, it has become clear that exosomal miRNAs play critical roles in mediating cell-cell communication, specifically between immune cells, endothelial cells and cancer cells (12–17). These findings provide evidence that exosomal miRNAs are required for cell-cell communication in various physiological and pathological conditions, although the contribution of extracellular miRNAs to cancer metastasis remains largely unknown (9). Here, we first demonstrated that horizontal transfer of exosomal miR-210 from metastatic cancer cells could dictate the microenvironmental endothelial cells to the benefit of the cancer cells, which contributed to cancer metastasis. Preventing the expression of

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[3] The abbreviations used are: miRNA, microRNA; nSMase2, neutral sphingomyelinase 2; KD, knockdown; luc, luciferase; HUVEC, human umbilical cord vein endothelial cell; nSMase2-OE, nSMase2-overexpressing cancer cells; qRT-PCR, quantitative RT-PCR.

nSMase2 in metastatic cancer cells abrogates the metastatic ability of cancer cells to target lung tissues, whereas reconstitution via the administration of exosomes isolated from metastatic cancer cells rescued this phenomenon. In this context, the number of endothelial cells in inoculated tumors was proportional to the expression level of nSMase2 in cancer cells. In fact, exosomes derived from a metastatic cancer cell line enhanced the capillary formation and migration of endothelial cells *in vitro*. Interestingly, the expression profiles of exosomal miRNAs obtained from metastatic cancer cells demonstrated that a set of angiogenic miRNAs were highly concentrated in these exosomes. One of them, miR-210, enhanced the angiogenesis through the suppression of specific target gene, which resulted in enhanced angiogenesis. These results revealed that cancer cells provide nSMase2-regulated exosomal miRNAs to endothelial cells to promote their metastatic initiation efficiency.

EXPERIMENTAL PROCEDURES

Reagents—Goat polyclonal anti-Alix (Q-19; sc-49268) and donkey anti-goat IgG (HRP; sc-2020) were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-HSP70, clone 7/HSP70 (610607), and mouse monoclonal ant-human CD63 antibody (556019) were purchased from BD Biosciences. Rabbit polyclonal anti-CD31 antibody (ab28364) was from Abcam. Peroxidase-labeled anti-mouse antibodies were purchased from GE Healthcare (NA931V). GW4869 was purchased from Calbiochem (Darmstadt, Germany). Geneticin and puromycin were purchased from Invitrogen.

Cell Culture—4T1 cells, a mouse breast cancer cell line, MCF7, non-metastatic breast cancer cells, and MCF10A, normal mammary epithelial cells, were obtained from the American Type Culture Collection (Manassas, VA). MDA-MB-231-D3H1 and MDA-MB-231-D3H2LN, a metastatic human breast cancer cell line, were obtained from Xenogen. 4T1, MCF7, MDA-MB-231-D3H1, and MDA-MB-231-D3H2LN were cultured in RPMI containing 10% heat-inactivated FBS and antibiotic-antimycotic (Invitrogen) at 37 °C in 5% CO_2. Human umbilical cord vein endothelial cells (HUVECs) were purchased from Lonza and cultured in EBM-2 BulletKit (Lonza) supplemented with 2% FBS.

Exosome Purification—Exosomes were purified by differential centrifugation as described previously (10). The exosome fraction was measured for its protein content using the Micro BCA protein assay kit (Thermo Scientific, Wilmington, DE).

Tube Formation Assay—HUVECs (100,000) cells were cultured on 150 μl of Matrigel (Sigma) in culture medium for 16 h in 24-well plate. The degree of tube formation was quantified by measuring the number of branches in five randomly chosen fields from each well using NIH ImageJ software. For rescue experiments, HUVECs were transfected using Dharmafect reagent (Dharmacon) according to the manufacturer's recommendations with anti-control or anti-miR-210 (Ambion). After 24 h of posttransfection, cells were seeded onto Matrigel as described above with 1 μg of exosome.

Establishment of Stable Cell Lines—A stable 4T1 and MDA-MB-231-D3H2LN nSMase2-modified cell lines that expressed mouse nSMase2 shRNA, human nSMase2 shRNA, and pCT-CD63-GFP were generated by selection with puromycin. A stable 4T1 and MDA-MB-231-D3H2LN cell lines that overexpresses human nSMase2 were generated by selection with geneticin. 4T1 cells or MDA-MB-231-D3H2LN were transfected with 0.5 μg of the vector at 90% confluency in 24-well dishes using a Lipofectamine LTX reagent in accordance with the manufacturer's instructions.

Co-culture Experiments—Well inserts for 24-well plates with a 0.4-μm pore-sized filter were purchased from BD and used following the manufacturer's instructions. 4T1 control cells, 4T1-nSMase2-KD cells, 4T1-siLuc cells, or 4T1-CD63-GFP cells (100, 000) were seeded into the well inserts. HUVECs (200, 000) were seeded into 24-well plates.

Confocal Microscopy—Confocal microscopy was done on an Olympus laser scanning microscope FV10i (Olympus). Filters used were 489–510 nm (GFP and Alexa Fluor 488) and 577–603 nm (Alexa Fluor 568).

Immunoblot Analysis—Exosomes were lysed in a 2% SDS buffer, and equal amounts of protein were loaded onto an SDS-PAGE gel. Anti-Alix (1:200), anti-HSP70 (1:1,000), and anti-CD63 (1:200) were used as primary antibodies. The dilution ratio of each antibody is indicated in *parentheses*. Two secondary antibodies (peroxidase-labeled anti-goat and anti-mouse antibodies) were used at a dilution of 1:2000. Bound antibodies were visualized by chemiluminescence using the ImmunoStar LD (290-69904) (Wako), and luminescent images were analyzed by a LuminoImager (LAS-3000; Fujifilm, Inc.). Only gels for CD63 (BD Biosciences) detection were run under non-reducing conditions.

Plasmids—psiRNA-LucGL3 was purchased from InvivoGen. Knockdown shRNA vector for human and mouse nSMase2 were purchased from TaKaRa Bio. A full-length human nSMase2 cDNA was cloned into pIRES2-EGFP vector (Clontech). Primary miR-210 were PCR-amplified from human genomic DNA and cloned into the downstream of CMV promoter in pIREShyg3 (Takara Bio). The sensor vector for miR-210 was constructed by introducing tandem binding sites with perfect complementarity to miR-210, separated by a four-nucleotide spacer into the XhoI site of psiCHECK2 (Promega). The sequences of the binding site are as follows: 5′-TTCTCGAGTTTCAGCCG-CTGTCACACGCACAGTTACGCGTTTTCAGCCGCTGT-CACACGCACAGTTCTCGAGTT-3′ (sense) and 5′-AACTC-GAGAACTGTGCGTGTGACAGCGGCTGAAAACGCGT-AACTGTGCGTGTGACAGCGGCTGAAACTCGAGAA-3′ (antisense). The "seed" sequence of miR-210 is *underlined*. In a mutated miR-210 sensor vector, the seed sequence, ACACGCA, was displaced with TGTGCGT. All of the plasmids were verified by DNA sequencing.

Isolation of RNAs—Isolation of exosomal and cellular RNAs was performed using the miRNeasy Mini Kit (Qiagen). Exosome or cell lysate was diluted with 1 ml of Qiazol solution. Subsequent extraction and filter cartridge work were carried out according to the manufacturer's protocol.

mRNA and miRNA Expression Analysis—The method for qRT-PCR has been described previously (10). PCR was carried out in 96-well plates using the 7300 Real Time PCR system (Applied Biosystems). All reactions were done in triplicate. All TaqMan MicroRNA assays were purchased from Applied Bio-

systems. RNU6 was used as an invariant control for the cells. Gene expression was analyzed using Taqman gene expression assays except primary miR-210 (Applied Biosystems). The expression levels of primary miR-210 and β-actin were measured by qRT-PCR using a SYBR Green PCR Master Mix (Invitrogen). Primer sequences are as follows (shown 5′ to 3′): primary miRNA-210, GACTGGCCTTTGGAAGCTCC (forward) and ACAGCCTTTCTCAGGTGCAG (reverse); β-actin, GGCACCACCATGTACCCTG (forward) and CACGGAGTACTTGCGCTCAG (reverse).

Nanoparticle Tracking Analysis—Nanoparticle tracking analysis was carried out using the Nanosight LM10-HS system (NanoSight) on exosomes resuspended in PBS and were further diluted for analysis. The results are presented as the average ± S.E. of three independent experiments.

Phase Contrast Electron Microscopy—A drop of the sample was put on a copper grid and coated with a carbon film with holes in it. Most of the liquid was removed with blotting paper, leaving a thin film stretched over the holes. The specimen was instantly shock-frozen by plunging into liquid ethane, which was cooled to 90 K by liquid nitrogen into a temperature-controlled freezing unit (Zeiss, Oberkochen, Germany). The remaining ethane was removed with blotting paper, and the specimen was transferred to the electron microscope. The phase plate was prepared from amorphous carbon films. The films were deposited by vacuum evaporation (JEOL JEE-400) on a freshly cleaved mica surface. For observation at 300-kV acceleration voltage, the film thickness corresponding to the *p*/two-phase plate was approximately 32 nm. At that thickness, the transparency of 300-kV acceleration electrons was estimated to be 70%. After preparation, the films were floated on the water's surface and then transferred to a molybdenum aperture with several holes 50-lm in diameter, which resulted in a cut-off frequency for special resolution of 0.5 nm. A hole approximately 0.5 lm in diameter in the center of the carbon film was used by a focused ion beam machine (JEOL JFIB-2000).

PKH67-labeled Exosome Transfer—Purified exosomes derived from 4T1 conditioned medium were labeled with a PKH67 green fluorescent labeling kit (Sigma-Aldrich). Exosomes were incubated with 2 μM PKH67 for 5 min, washed four times using 100-kDa filter (Microcon YM-100, Millipore) to remove excess dye, and incubated with HUVECs at 37 °C.

Microarray Analysis—To detect the miRNAs in exosomes and cells derived from HEK293, MCF10A, MCF7, and MDA-MB-231, 100 ng of total RNA was labeled and hybridized using a human microRNA microarray kit (Agilent Technologies) according to the manufacturer's protocol (protocol for use with Agilent MicroRNA microarrays, version 1.5). Hybridization signals were detected using a DNA microarray scanner (Agilent Technologies), and the scanned images were analyzed using Agilent Feature Extraction software.

Mouse Studies—Animal experiments in this study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. Five- to seven-week-old female Balb/c athymic nude mice (CLEA Japan, Shizuoka, Japan) or SCID Hairless Outbred mice (Charles River Laboratories, Kanagawa, Japan) were anesthetized by exposure to 3% isoflurane for injections and *in vivo* imaging. We injected 4T1- or MDA-MB-231-D3H2LN-nSMase2-modified cells bilaterally into the subcutaneous (2×10^6 cells were injected in 100-μl volume PBS) or mammary fat pad (2×10^6 cells were injected in 50-μl volume Matrigel diluted with PBS) of anesthetized mice. We monitored mammary tumor growth by regular measurements using a digital caliper. After 3 to 4 weeks, we killed mice and determined metastasis in lungs by *ex vivo* or *in vivo* imaging. We carried out lung colonization assays by injecting 1×10^6 4T1-control or 4T1-nSMase2-KD cells (suspended in 100 μl of PBS) into the lateral tail vein. Lung colonization was studied and determined by *in vivo* luminescence imaging. For rescue experiment, 4T1-nSMase2-KD cells (2×10^6 cells suspended in 100 μl of PBS) were subcutaneously injected. After 4 days of implantation, 1 μg of exosome was injected intratumoraly (100 μl in PBS) every other day for up to 18 days. Metastasis occurrence was determined by *in vivo* luminescence. For *in vivo* imaging, the mice were administered D-luciferin (150 mg/kg, Promega) by intraperitoneal injection. Ten minutes later, photons from animal whole bodies were counted using the IVIS imaging system (Xenogen) according to the manufacturer's instructions. Data were analyzed using LIVINGIMAGE software (version 2.50, Xenogen).

Statistics—Statistical analyses were performed using the Student's *t* test.

RESULTS

nSMase2 Regulates Cancer Cell Metastasis—In a previous study, we have described how miRNAs are released through ceramide-dependent secretory machinery via the exosome (10). Specifically, we demonstrated that blocking the activity of nSMase2 resulted in reduced miRNA secretion and that nSMase2 overexpression led to increased levels of extracellular miRNAs (10, 11). In addition, we found that the expression level of nSMase2 was higher in cancer cells than that in non-cancer cells (Fig. 1*A*, *upper panel* and supplemental Fig. 1*A*). Furthermore, secretion level of exosome show correlation with the expression level of nSMase2 (Fig. 1*A*, *lower panel*, and supplemental Fig. 1*B*), suggesting that malignant cancer cells secrete more exosomes than non-cancer cells through the regulation of nSMase2. We confirmed that breast cancer cells secreted around 100-nm size of vesicles with a consistent size and uniform expression of known exosome marker, CD63 and HP70 (supplemental Fig. 1*C*) (18). Purified exosomes has also been shown by phase contrast electron microscopy and found that their size observed to be 90 ± 11.7 nm in diameter ($n = 13$) (Fig. 1*B*). To determine the role of nSMase2 in cancer cell malignancy, we employed 4T1 cells, which are mouse mammary tumor cells with a high tumorigenic and metastatic ability. Both stable nSMase2-knockdown and nSMase2-overexpressing 4T1 cells were generated (supplemental Figs. 1, *D* and *E*) and inoculated into mammary fat pad of the mice, and the tumors were subsequently evaluated for their metastatic colonization capacity in lung tissue. The expression of secretory miR-16 (supplemental Fig. 2*A*), which is known to abundantly existed in exosome, as well as exosome quantity, as determined by immunoblotting for exosome markers, HSP70 and Alix (supplemental Fig. 2*B*), protein concentration (supplemental Fig. 2*C*), and nanopar-

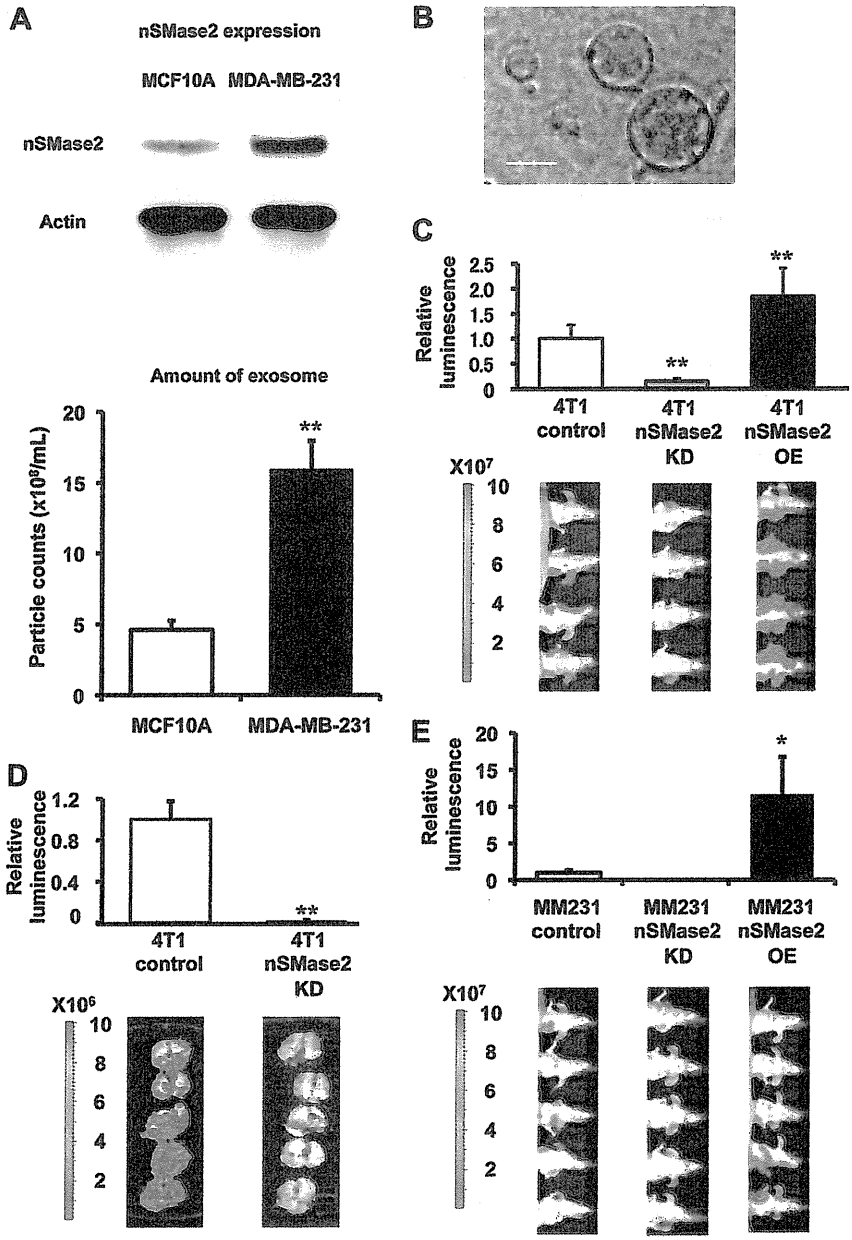

FIGURE 1. **nSMase2 regulates cancer cell metastasis.** *A*, the expression level of nSMase2 protein in (*upper panel*) and secretion level of exosome from (*lower panel*) MCF10A and MDA-MB-231 cells. The same number of cells was seeded. *Error bars* are presented as the mean S.E. ($n = 3$). **, $p < 0.005$, as compared with MCF10A cells. *B*, phase-contrast electron microscopy was used to image resuspend exosome pellets. *Scale bar*, 100 nm. *C*, bioluminescence quantification of lung metastasis by 4T1-control cells, 4T1-nSMase2-KD cells or 4T1-nSMase2-OE cells. Each *error bar* is presented as the mean S.E. ($n = 4$). **, $p < 0.005$, as compared with 4T1-control cells. *D*, luciferase activity in the lung, which was used to represent lung metastasis, was recorded for each mouse. Lung images from different mice are shown. Each *error bar* is presented as the mean S.E. ($n = 5$). **, $p < 0.005$, as compared with 4T1-control cells. *E*, bioluminescence quantification of metastasis by parental MDA-MB-231-D3H2LN (*MM231-control*) cells, MDA-MB-231-D3H2LN-nSMase2-OE (*MM231-nSMase2-OE*) cells, or MDA-MB-231-D3H2LN-nSMase2-KD (*MM231-nSMase2-KD*) cells. Each *error bar* is presented as the mean ± S.E. ($n = 5$). *, $p < 0.05$, as compared with MM231-control cells.

ticle tracking analysis (supplemental Fig. 2*D*), decreased in nSMase2-knockdown cancer cells (4T1-nSMase2-KD cells) but increased in nSMase2-overexpressing cancer cells (4T1-nSMase2-OE cells). However, the expression of intracellular miRNAs was not altered in either of these established cell types (supplemental Figs. 2*A* and 3). After the orthotopic inoculation of these cell lines into mammary fat pad, we found that nSMase2 silencing in parental 4T1 breast cancer cells significantly decreased lung metastatic colonization (Fig. 1*C*), and *in vivo* imaging and histological observation revealed a significant decrease in the total number of metastatic nodules in nSMase2-knockdown lung tumors (Fig. 1*D* and supplemental Fig. 4*A*). In contrast, the overexpression of nSMase2 in 4T1 cells enhanced the metastatic capacity of these tumors (Fig. 1*C*). We also confirmed similar results using an orthotopic model of MDA-MB-231-D3H2LN cells, which are human breast cancer cells with a

high metastatic ability, overexpressing or inhibiting nSMase2 (Fig. 1*E*), which suggests that the alteration in expression level of nSMase2 leads to the change in metastatic ability of cancer cells. Interestingly, nSMase2 inhibition or overexpression in 4T1 cells did not significantly enhance or inhibit cellular proliferation, invasion, or migration *in vitro* (supplemental Fig. 4*B*) and did not increase the mammary tumor volume (supplemental Fig. 4, *C* and *D*). In addition, no significant differences were found in expression profiles of cellular or miRNAs isolated from these nSMase2-modified cell lines (supplemental Fig. 3). Moreover, no significant reduction in metastatic potential was observed in the lungs of animals intravenously injected with parental 4T1 cells or 4T1-nSMase2-KD cells, which excludes the possibility that nSMase2 disruption affected the recruitment capacity of cancer cells to metastatic tissues (supplemental Fig. 5). These results indicate that the effect of nSMase2 on metastasis was not simply due to its effect on the cancer cells themselves.

Endothelial Activation Regulated by nSMase2-mediated Exosome Promotes Cancer Cell Metastasis—Consistent with a role for nSMase2 in the initiation of metastasis, intratumor injection of exosomes isolated from parental 4T1 cells to non-metastatic 4T1-nSMase2-KD cells after orthotopical inoculation into mammary fat pad significantly enhanced their metastatic colonization (Fig. 2*A* and supplemental Fig. 6*A*), whereas the growth of the inoculated 4T1-nSMase2-KD tumor cells was unaffected (supplemental Fig. 6*B*). These results indicated that endogenous nSMase2 could act to enhance metastatic initiation through the secretion of exosomes. When examining the selective disadvantage provided by nSMase2 silencing in cancer cells, we noticed that blood vessels were difficult to detect in animals that received 4T1-nSMase2-KD cells (Fig. 2*B*, *left panel*). As a result, we hypothesized that tumors inoculated with nSMase2-knockdown cells would display reduced blood vessel densities upon microscopic visualization of the primary tumor after staining for the endothelial marker CD31. The imaging analysis revealed that primary tumors derived from 4T1-nSMase2-KD cells had significantly lower endothelial cell densities than did tumors derived from control cells (Fig. 2*B*, *right panel*, and 2*C*). In contrast, tumors derived from 4T1-nSMase2-OE cells displayed higher endothelial densities than did tumors derived from control cells (Fig. 2*B*, *right panel*, and 2*C*). In addition, there were increased numbers of endothelial cells in tumors derived from 4T1-nSMase2-KD cells that were subsequently injected with parental 4T1 cell-derived exosomes compared with control treatment (Fig. 2, *D* and *E*). Thus, these observations indicate that release of nSMase2-mediated exosome enhances endothelial cell density, whereas the inhibition of nSMase2 provides metastatic cells with a selective disadvantage for endothelial interactions and angiogenic progression.

Exosomes Derived from Metastatic Cancer Cells Enhances Activity of Endothelial Cells—We next sought to determine the cellular basis for nSMase2-regulated exosome-dependent angiogenesis. For this purpose, we first evaluated the effect of exosome from parental 4T1 cells in HUVECs. As a result, although cellular proliferation of HUVECs was slightly increased by the addition of 4T1 exosome (supplemental Fig. 7*A*), addition of purified exosomes derived from metastatic 4T1 cells enhanced not only tube formation in HUVECs, as assessed by the quantification of branch points (Fig. 3*A*), but also migration of HUVECs (Fig. 3*B*). Next, to determine whether exosomes secreted by metastatic breast cancer cells could be incorporated in a paracrine manner, we employed a co-culture system for HUVECs and 4T1 cells, in which the cells are separated by a membrane with a 0.4-μm pore size to prevent direct cell contact or the transfer of larger vesicles. In this experiment, we used 4T1 cells that had been transduced with a CD63-GFP fusion gene, and we analyzed GFP fluorescence present in HUVECs after 3 days of co-culture by confocal microscopy. These studies showed that exosomes could be transferred from breast cancer cells to endothelial cells during co-culture (Fig. 3*C*, *left panel*). However, the transfer of exosomes from cancer cells to endothelial cells was completely abolished by the addition of nSMase2 inhibitor, GW4869 that was reported to inhibit the secretion of exosome from cells (10, 19), to the 4T1-CD63-GFP cells (Fig. 3*C*: right panel). In addition, 4T1 exosomes labeled with the fluorescent dye PKH67 were cultured with HUVECs and were found to be internalized into endosome-like structures by endothelial cells (supplemental Fig. 7*B*). To confirm whether the exosomes from inoculated cancer cells were incorporated into endothelial cells *in vivo*, an immunohistochemical analysis was performed following the inoculation of 4T1-hCD63 cells *in vivo* (Fig. 3*D*). As shown in Fig. 3*D*, the CD31-positive cells (*green*) was co-localized with CD63 (*red*) signals in the tumor. These findings reveal that enhanced tube formation in endothelial cells is a key feature of metastatic breast cancer cell populations that is regulated in a humoral fashion by exosomes released from metastatic cancer cells.

Exosomal Angiogenic miRNAs from Cancer Cells Regulate Angiogenesis in Endothelial Cells—It is well known that angiogenic miRNAs regulate multiple endothelial cell functions and that nSMase2 is essential for miRNA secretion from cells (10, 20, 21). These reports, in addition to our findings described above, prompted us to evaluate the hypothesis that exosomal miRNAs from cancer cells are responsible for this phenomenon. To prove this hypothesis, we used 4T1 cells that had been transduced with a luciferase short hairpin RNA-overexpressing vector (4T1-siLuc). This established cell line secretes luciferase siRNA molecules with a nucleic acid sequence not present in the mammalian genome (supplemental Fig. 8*A*). To evaluate whether the transfer of luciferase siRNA occurred in the form of exosome transfer, we added GW4869 to co-cultured 4T1-siLuc cells and assessed the transfer of luciferase siRNA to HUVECs by qRT-PCR. Although we were able to measure luciferase siRNA in control-treated HUVECs, this siRNA sequence was minimally detected in HUVECs co-cultured with GW4869 treated 4T1-siLuc cells (Fig. 4*A*), which indicates that small RNAs, including not only siRNA but also miRNA, could in fact be transferred from cancer cells to endothelial cells during co-culture and that the transfer of small RNAs is mediated by exosome release regulated by nSMase2. We next sought to determine whether exosomal miRNAs could selectively regulate the angiogenesis of endothelial cells. To address this hypothesis, we performed a miRNA microarray analysis of the following four cell lines: metastatic breast cancer cells (MDA-MB-231); non-metastatic cancer cells (MCF7); normal mam-

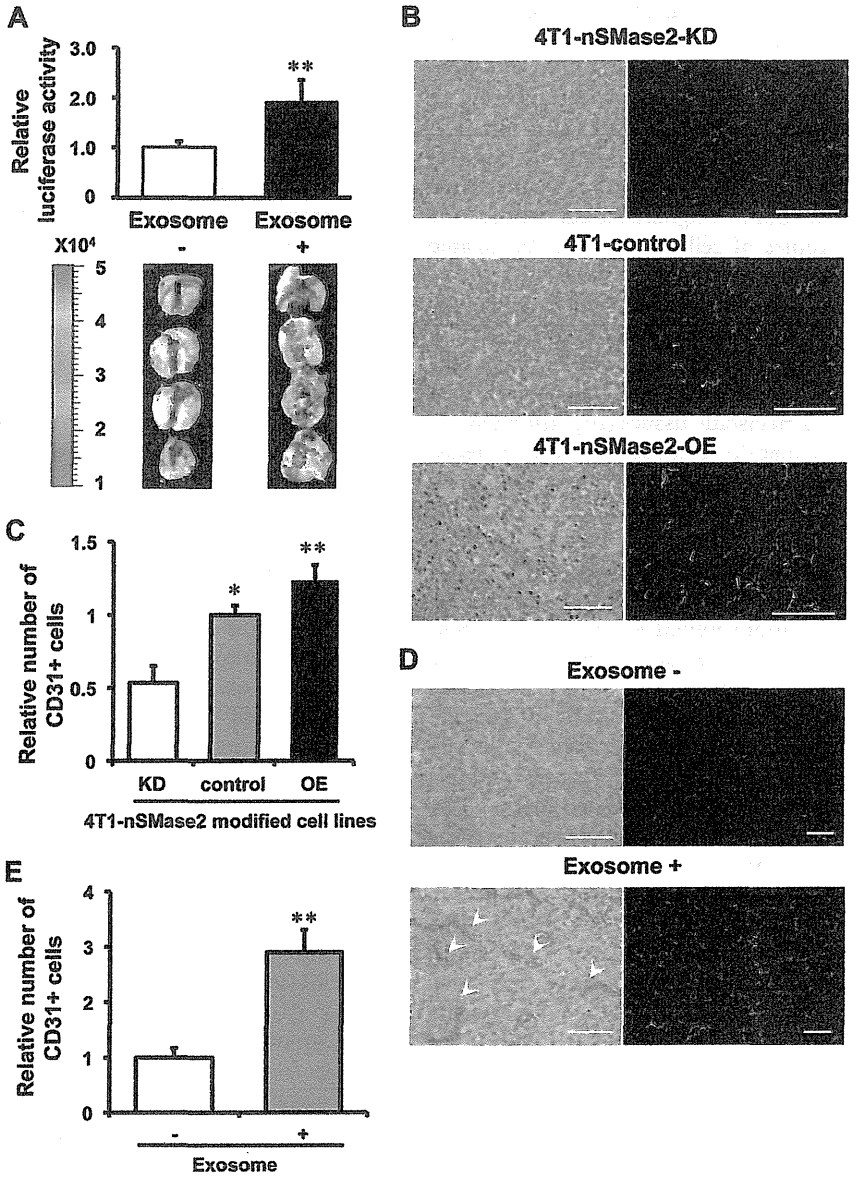

FIGURE 2. **Endothelial activation mediated by nSMase2 regulates cancer cell metastasis.** *A*, bioluminescence imaging of lung metastasis by 4T1-nSMase2-KD cells with or without the injection of exosomes isolated from parental 4T1 cells. Each *error bar* is presented as the mean ± S.E. ($n = 4$). **, $p < 0.005$, as compared with control injection. *B*, H&E of primary tumors isolated from parental 4T1-control cells, 4T1-nSMase2-KD cells or 4T1-nSMase2-OE cells (*left panel*). *Scale bars*, 100 μm for H&E. The endothelial cells were also evaluated using CD31 staining to detect blood vessels in tumors composed of parental 4T1 cells, 4T1-nSMase2-KD cells or 4T1-nSMase2-OE cells (*right panel*); *scale bars*, 100 μm. *C*, angiogenesis determined using CD31 staining which shown in *B* to detect blood vessels in tumors composed of parental 4T1 cells, 4T1-nSMase2-KD cells, or 4T1-nSMase2-OE cells, as above; $n = 4$ for each group. Each *error bar* is presented as the mean ± S.E. ($n = 4$). *, $p < 0.05$; **, $p < 0.005$, as compared with 4T1 control. *D*, H&E staining of primary tumors isolated from mice that received PBS or an injection of exosomes from parental 4T1 cells following the transplantation of 4T1-nSMase2-KD cells (*left panel*). *Arrowheads* show red blood cells in vascular structure. *Scale bars*, 100 μm for H&E. The endothelial cells were also evaluated using CD31 staining to detect blood vessels in tumors composed of 4T1-nSMase2-KD cells with or without exosome injection (*right panel*); *scale bars*, 200 μm. *E*, angiogenesis determined using CD31 staining, which was shown in *D* to detect blood vessels in tumors composed of 4T1-nSMase2-KD cells with or without exosome, as above; $n = 4$ for each group. Each *error bar* is presented as the mean ± S.E. ($n = 4$). **, $p < 0.005$, as compared with control injection.

mary epithelial cells (MCF10A); and human embryonic kidney cells (HEK293). The microarray analysis of miRNA populations in exosomes isolated from these cell lines were performed using a miRNA microarray (Fig. 4*B*). Interestingly, some of the exosomal miRNAs that were highly enriched in the metastatic cancer cell line are known to regulate angiogenesis in endothelial cells (22). One of these miRNAs, miR-210, which is well known as an angiogenic miRNA, and its expression was correlated with poor prognosis in breast cancer (23, 24). Moreover, a recent report showed that high expression levels of miR-210 in plasma are associated with the presence of tumor in patients with breast cancer and with trastuzumab resistance in patients with HER2-positive breast cancer (25). In addition, the expression level of miR-210 was significantly higher in breast cancer patients with lymph node metastasis than in breast cancer patients without lymph node metastasis (25). From our data

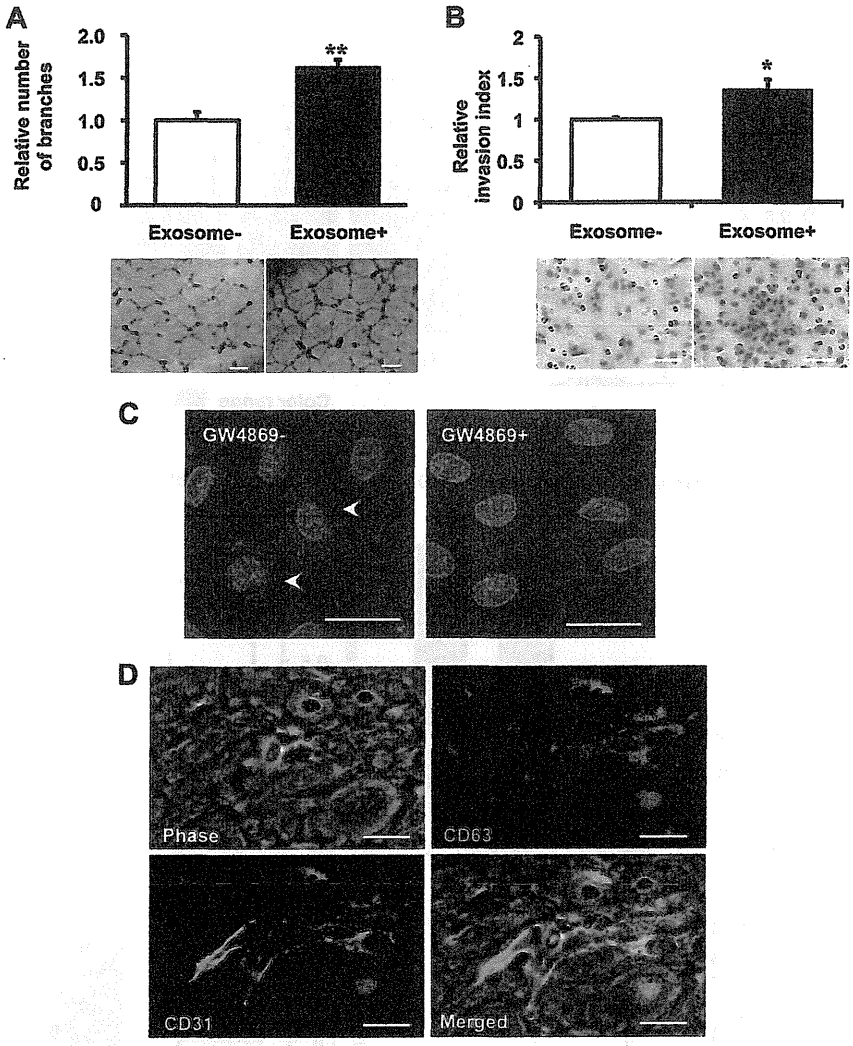

FIGURE 3. **Exosomes derived from metastatic cancer cells enhances activity of endothelial cells.** *A*, capillary tube formation in endothelial cells seeded onto Matrigel following the addition of exosomes from parental 4T1 cells. A representative image at 16 h after plating is shown, including the quantification of the average number of branches at 16 h after plating. The *scale bar* indicates 500 μm. *B*, the effect of exosome on HUVEC migration was determined by Transwell migration assay. A representative image at 48 h after plating is shown, including the quantification of the average number of migrated HUVECs at 48 h after plating. The *scale bar* indicates 100 μm. *C*, an *in vitro* co-culture system was used, whereby 4T1 cells were seeded in the *top compartment* and separated from HUVECs in the *bottom compartment* by a porous membrane. 4T1 cells (*top compartment*) were transduced with a CD63-GFP vector and co-cultured with HUVECs (*bottom compartment*). *Scale bars*, 100 μm. *D*, immunostaining of CD31 (*green*) and CD63 (*red*) on 4T1-hCD63 inoculated tumor. The *scale bar* indicates 10 μm. CD63 is co-localized with CD31-positive endothelial cells.

and previous reports, because the contribution of exosome against cancer metastasis was mediator of endothelial activation, we postulated that exosomal miR-210 might be one of the regulators in exosome for the angiogenesis around the cancer cells. Indeed, miR-210 expression in exosome was higher in malignant cancer cells than that in non-malignant cancer cell or non-cancer cells (Fig. 4*C*). To confirm that exosomal miR-210 were down-regulated in nSMase2-impaired cancer cells, we performed a qRT-PCR analysis for these cells. As shown in Fig. 4*D*, the expression of exosomal miR-210 was down-regulated in nSMase2 knockdown cells when compared with control cells, although the cellular levels of the miRNAs were not altered (supplemental Fig. 8*B*). Moreover, we performed a co-culture experiment using 4T1-nSMase-KD cells or parental 4T1 cells with HUVECs and then measured the expression of miR-210 in the HUVECs. Co-culture with parental 4T1 cells, compared with 4T1-nSMase-KD cells, led to the higher detection of miR-210 in HUVECs (Fig. 4*E*), indicating that exosomal miR-210 from metastatic cancer cells transfer to recipient endothelial cells. Then, we employed this co-culture system to study the effects of exosomes isolated from parental 4T1 cells on the expression of the established miR-210 target gene, ephrin-A3 (26). The presence of parental 4T1 cells reduced the expression level of ephrin-A3 in HUVECs compared with 4T1-nSMase2-KD cells (Fig. 4*F*). To exclude the possibility that the exosome from cancer cells itself induces the endogenous expression of miR-210 in HUVECs, we quantified the expression of primary miR-210 in HUVECs co-cultured with parental 4T1 cells or 4T1-nSMase-KD cells. As shown in supplemental Fig. 8*C*, we did not find any difference of primary miR-210

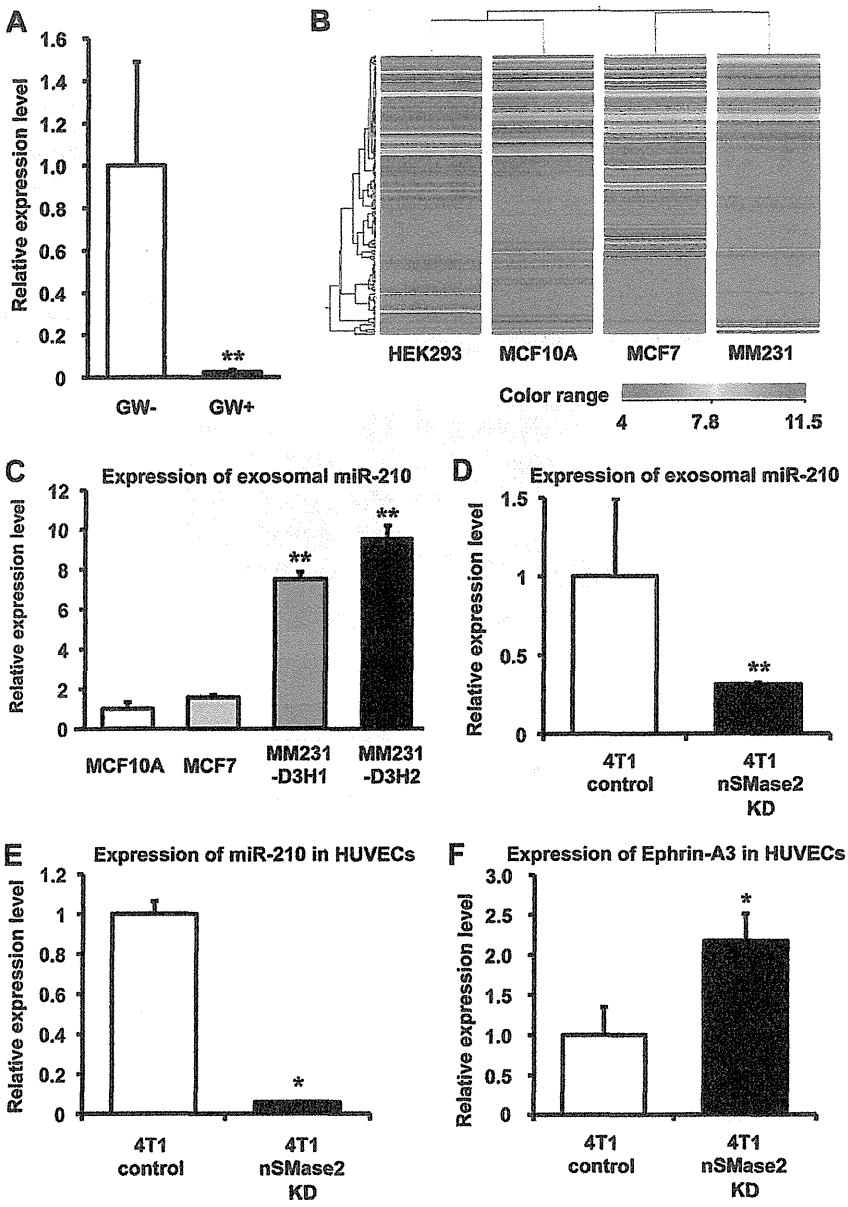

FIGURE 4. **Exosomal angiogenic miRNAs from cancer cells regulate angiogenesis in endothelial cells.** *A*, 4T1-siLuc cells were treated with 10 μM GW4869 at the start of the co-culture for a total of 48 h ($p < 0.001$). Each *error bar* is presented as the mean ± S.E. ($n = 3$). **, $p < 0.005$, as compared with control. *B*, heat map showing expression levels of the exosomal miRNAs isolated from HEK293, MCF10A, MCF7, and MDA-MB-231. *Blue* to *red*, color range gradient of mean abundance. *C*, the expression level of miR-210 in exosome isolated from MCF10A, MCF7, MDA-MB-231-D3H1 (MM231-D3H1), or MDA-MB-231-D3H2LN (MM231-D3H2) cells. Each *error bar* is presented as the mean ± S.E. ($n = 3$). **, $p < 0.005$, as compared with MCF10A. *D*, expression of exosomal miR-210 in exosomes isolated from parental 4T1 cells or 4T1-nSMase2-KD cells. Each *error bar* is presented as the mean ± S.E. ($n = 4$). **, $p < 0.005$, as compared with 4T1-control cells. *E*, HUVECs were co-cultured with parental 4T1 cells or 4T1-nSMase2-KD cells for 48 h. RNA was isolated from the HUVECs at 48 h after the start of co-culture, and the expression of exosomal miR-210 in the HUVECs was analyzed by qRT-PCR. Each *error bar* is presented as the mean ± S.E. ($n = 3$). *, $p < 0.05$, as compared with 4T1 control cells. *F*, parental 4T1 cells or 4T1-nSMase2-KD cells were co-cultured with HUVECs for 48 h, and the expression levels of ephrin-A3 (target of miR-210) were analyzed by qRT-PCR. Each *error bar* is presented as the mean ± S.E. ($n = 3$). *, $p < 0.05$, as compared with 4T1-control cells.

expression level between HUVECs co-cultured with parental 4T1 cells or 4T1-nSMase-KD cells, although the expression of primary miR-210 levels was induced 20-fold above basal levels by desferrioxamine, which is an iron chelator and known to induce the expression of hypoxia inducible factor-1α (27), treatment compared with untreated cells (supplemental Fig. 8*D*). Taken together, these results suggest that the enhanced angiogenesis mediated by exosomes isolated from metastatic cancer cells is due to the presence of angiogenic miRNAs within the exosomes.

Exosomal miR-210 Enhanced Angiogenic Activity in Endothelial Cells in Vitro—To show the direct evidence that exosomal miR-210 released from cancer cells contributed to the enhancement of endothelial function in HUVECs, we collected miR-210 enriched exosome, which was isolated from miR-210 transiently transfected 4T1 cells. After the transfection of miR-210

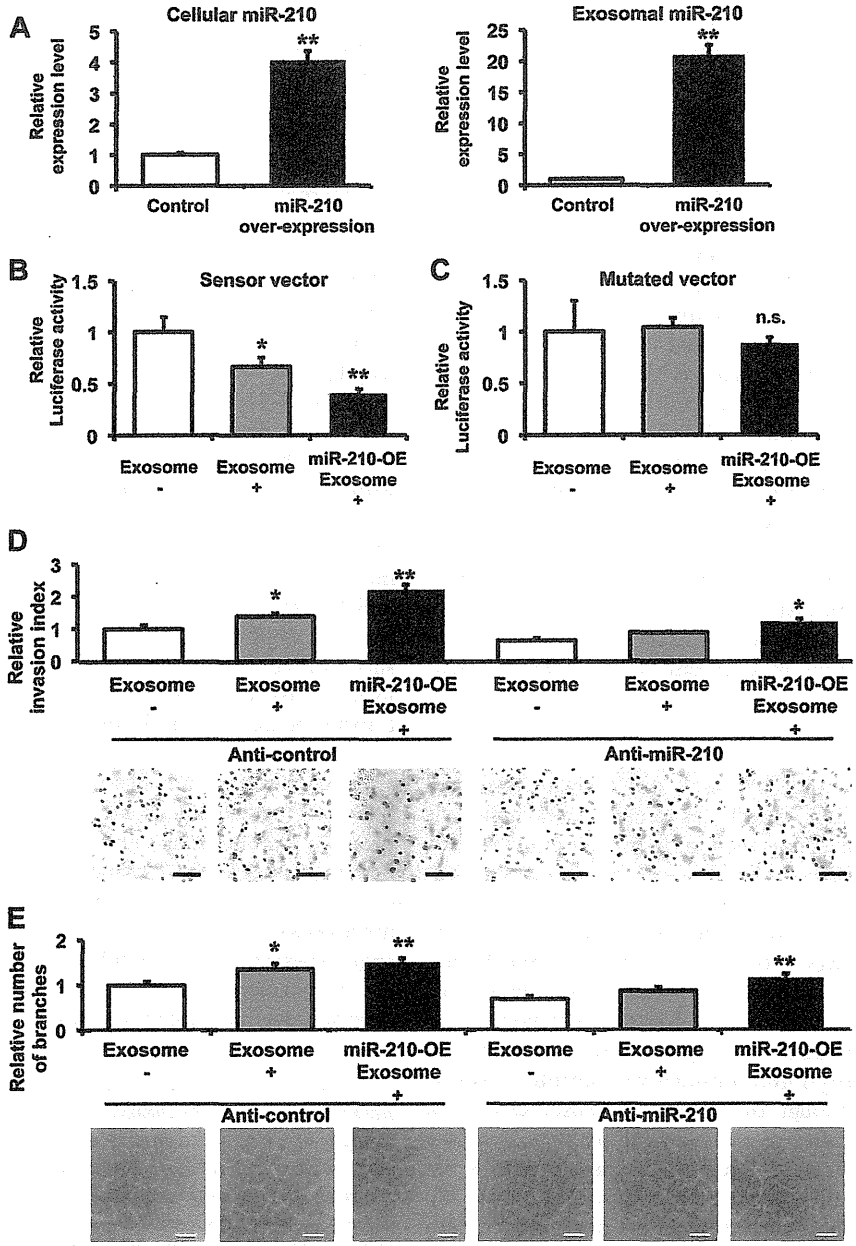

FIGURE 5. **Exosomal miR-210 from cancer cells enhanced the angiogenesis in endothelial cells.** *A*, the expression level of miR-210 in the cells (*left panel*) and exosome (*right panel*) from miR-210 overexpressing cells and control vector transfected cells. Each *error bar* is presented as the mean ± S.E. ($n = 3$). **, $p < 0.005$, as compared with control. *B*, Exosome derived from 4T1 cells suppressed the luciferase activity of the sensor vector. HUVECs transfected with an miR-210 sensor vector were used as recipient cells. The recipient cells were incubated in an miR-210-enriched exosome, control exosome, or PBS. After a 1-day incubation, a luciferase reporter assay was performed. The values on the *y* axis are depicted relative to the normalized luciferase activity of control PBS-treated cells, which is defined as 1. Each *error bar* is presented as the mean ± S.E. ($n = 5$). *, $p < 0.05$; **, $p < 0.005$, as compared with control. *C*, exosome did not reduce the luciferase activity of the mutated sensor vector. HUVECs transfected with the mutated miR-210 sensor vector were used as recipient cells. The recipient cells were incubated in an miR-210-enriched exosome, control exosome, or PBS. The luciferase assay was carried out as described above. The values on the *y* axis are depicted relative to the normalized *Renilla* luciferase activity of control cells, which is defined as 1. Each *error bar* is presented as the mean ± S.E. ($n = 4$). *n.s.* represents not significant. *D*, the transfection of anti-miR-210 to HUVECs inhibited the induction of capillary formation by exosomes derived from 4T1 cells. Following transfection with 3 nM of the miR-210 inhibitory molecule (anti-miR-210) or a control molecule (anti-NC), the HUVECs were incubated for 1 day, and these cells were then assessed using the migration assay with miR-210-enriched exosomes, control exosomes, or PBS. A representative image at 48 h after plating is shown, including the quantification of the average number of migrated HUVECs at 48 h after plating. Each *error bar* is presented as the mean ± S.E. ($n = 3$). *, $p < 0.05$; **, $p < 0.005$ as compared with PBS treatment. The *scale bar* indicates 100 μm. *E*, capillary tube formation in endothelial cells seeded onto Matrigel following the addition of miR-210-enriched exosomes, control exosomes, or PBS. A representative image at 16 h after plating is shown, including the quantification of the average number of branches at 16 h after plating. Each *error bar* is presented as the mean ± S.E. ($n = 3$). *, $p < 0.05$; **, $p < 0.005$ as compared with PBS treatment. The *error bar* indicates 500 μm.

expression vector to 4T1 cells, its expression was increased not only in the cells (Fig. 5*A*, *left panel*) but also in the exosomes (Fig. 5*A*, *right panel*). To confirm whether the transferred miR-210 are functional in the recipient HUVECs or not, we performed an miRNA-responsive reporter assay. We implemented luciferase analyses using a sensor vector harboring *Renilla*

luciferase tandemly fused with miR-210 antisense sequence in the 3′-UTR. As shown in Fig. 5*B*, the normalized *Renilla* luciferase activities were reduced by the addition of exosome derived from 4T1 cells. Furthermore, miR-210-enriched exosome suppressed luciferase activity more effective than original exosome (Fig. 5*B*). In contrast, we did not detect any changes of luminescence by using a mutated vector instead of the intact sensor vector (Fig. 5*C*), indicating that exosomal miR-210 transferred and functional in recipient endothelial cells. Although the cellular proliferation of HUVECs was only slightly induced by the addition of miR-210-enriched exosome (supplemental Fig. 9), migration and capillary formation of HUVECs were significantly enhanced by the addition of miR-210-enriched exosome (Fig. 5, *D* and *E*). Though miR-210 inhibitory molecule (anti-miR-210) inhibited capillary formation and migration of HUVECs treated by exosome, and this inhibition was partially overcome by the addition of miR-210 enriched exosome (Fig. 5, *D* and *E*), indicating that miR-210 in exosome had a function to modulate endothelial activation. Taken together, these results illustrate that the transfer of exosomal miR-210 from metastatic cancer cells to endothelial cells is regulated by cancer cell nSMase2 expression and the activation of endothelial cells to overcome their niche for their benefit.

DISCUSSION

Our data indicate that nSMase2 can activate exosomal miRNA secretion, which contributes to cancer cell metastasis through the induction of angiogenesis in the tumor microenvironment. These findings establish a key role for cancer cell-endothelial cell interactions for the initiation of metastasis.

Open questions remain regarding the physiological importance of exosome, however, the evidences for the contribution of exosome in cancer malignancy have been accumulating. For instance, exosomes from highly metastatic melanoma cells increased the metastatic behavior of primary tumors by educating bone marrow progenitors through the receptor tyrosine kinase MET (28). Although the number of exosomes did not differ based on clinical stage of melanoma patients, exosome protein concentrations were higher in subjects with stage 4 disease compared with other stages and to normal controls (28). Furthermore, exosome from metastatic breast cancer cells induced the mobilization of a population of neutrophil immune cells (29). Thus, all of these studies showed the possible involvement of angiogenic exosome to promote cancer metastasis. In the present study, we have found that exosomal angiogenic miRNAs, such as miR-210, regulate the metastatic ability of cancer cells. Considering that the circulating miR-210 level was increased in the serum of cancer patients with malignant breast cancer (25), exosomal angiogenic miR-210 might be one of the key factors for the tumor angiogenesis in the pathophysiological condition.

It has been known that nSMase2, which generates ceramide production in the cells, regulates multiple cellular activities in the cells via ceramide signaling. For instance, nSMase2 has been reported to act as a growth suppressor in MCF7 cells (30). On the contrary, nSMase2 was activated by Urokinase-type plasminogen activator triggering interaction of integrin $\alpha_v\beta_3$, Urokinase-type plasminogen activator receptor, and matrix metalloproteinases, resulting in the induction of cellular proliferation (31). These reports suggest that the effects of nSMase2 up-regulation or down-regulation depend on the cellular origin and situation. In this article, we clearly showed that modulation of nSMase2 affect the exosome production from mouse mammary tumor cells lines 4T1 cells and human breast cancer cell lines, MDA-MB-231 cells. To further understand the exosome-mediated cancer progression, it is essential to examine whether nSMase2 regulate the exosome production in every types of cancer cells or not.

miRNAs were known to affect the expression of multiple target genes. For this reason, we could not rule out the possibility that miR-210 overexpression induced the angiogenic factors in exosome. In this work, we prepared the "miR-210-enriched exosome" by transient transfection and collected the exosome within 2 day after the transfection of miR-210 vector to try to avoid the effect of miR-210 in cancer cells. In addition, the effect of exosome was partially cancelled by the introduction of miR-210 inhibitor in HUVECs (Fig. 5, *D* and *E*). Furthermore, miR-210-enriched exosome overcome the inhibitory activity of miR-210 inhibitor in HUVECs (Fig. 5, *D* and *E*). These results suggest that the miR-210 in exosome from cancer cells can be incorporated in endothelial cells cells via exosomes, and this transferred miR-210 itself suppress their target genes, resulting in the activation of endothelial cells.

In conclusion, we propose that cancer cells provide nSMase2-regulated exosomal miRNAs to endothelial cells to promote their metastatic initiation efficiency. This work is the first to connect cancer metastasis to the nSMase2-mediated exosome *in vivo* and demonstrates that exosome-mediated metastasis occurs via the enhancement of microenvironmental angiogenesis by exosomal miRNAs. To understand the molecular mechanism of this on-demand system should also shed light on novel approaches for cancer therapy through the inhibition of angiogenesis.

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REVIEW ARTICLE
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Trash or Treasure: extracellular microRNAs and cell-to-cell communication

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Circulating RNAs in human body fluids are promising candidates for diagnostic purposes. However, the biological significance of circulating RNAs remains elusive. Recently, small non-coding RNAs, microRNAs (miRNAs), were isolated from multiple human body fluids, and these "circulating miRNAs" have been implicated as novel disease biomarkers. Concurrently, miRNAs were also identified in the extracellular space associated with extracellular vesicles (EVs), which are small membrane vesicles secreted from various types of cells. The function of these secreted miRNAs has been revealed in several papers. Circulating miRNAs have been experimentally found to be associated with EVs; however, other types of extracellular miRNAs were also described. This review discusses studies related to extracellular miRNAs, including circulating miRNAs and secreted miRNAs, to highlight the importance of studying not only secreted miRNAs, but also circulating miRNAs to determine the contribution of extracellular miRNAs especially in cancer development.

Keywords: circulating microRNA, exosomes, extracellular vesicles, extracellular microRNA, secretory microRNA, cell-to-cell communication

INTRODUCTION

Circulating RNAs have been isolated from human body fluids (Kamm and Smith, 1972; Fleischhacker and Schmidt, 2007). Javillier and Fabrykant (1931) reported the first discovery of circulating nucleic acids in 1931, before Watson and Crick (1953) reported the structure of DNA as a double helix. Furthermore, Mandel and Metais (1947) permitted ribonucleic acid and deoxyribonucleic acid to be separately measured. Since then, many researchers have attempted to use circulating RNA as disease biomarkers; however, the origins and meanings of circulating RNA are poorly understood.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate multiple phenomena, including development, organogenesis, and homeostasis (Ebert and Sharp, 2012). The mis-expression of miRNAs results in the onset of diseases, such as immune disease, cardiovascular disease, neurological disease, and cancer (Mendell and Olson, 2012). In 2007, the Lötvall group demonstrated that miRNAs were contained inside exosomes (Valadi et al., 2007), which are small membranous vesicles derived from the endosome (Raposo and Stoorvogel, 2013). Since the discovery of miRNAs in exosomes, several reports confirmed the existence of miRNAs in apoptotic bodies (Zernecke et al., 2009), high-/low-density lipoprotein (HDL/LDL; Vickers et al., 2011), and RNA-binding proteins (Arroyo et al., 2011; Turchinovich et al., 2011). Other studies have shown the existence of circulating miRNAs in human serum, including the serum from pregnant women (Chim et al., 2008) and cancer patients (Lawrie et al., 2008). Researchers have identified placental-specific miRNAs in the serum from pregnant women, which clearly disappeared after childbirth, indicating that circulating miRNAs reflect the status of the individual (Chim et al., 2008). Similarly, cancer-associated miRNAs were higher in the serum from cancer patients than in the serum from healthy individuals, indicating that circulating miRNAs can be used as biomarkers to monitor the existence of cancer cells in patients (Lawrie et al., 2008). These reports also demonstrated the stability of circulating miRNAs in the blood, despite the presence of large amounts of RNase (Reddi and Holland, 1976). Since the discovery of miRNAs in blood, many researchers have confirmed the existence of miRNA in a variety of other human body fluids, such as serum, plasma, saliva, breast milk, urine, and cerebrospinal fluid, among others (Kosaka et al., 2010a).

In this review, we chose miRNAs that were reported to have functions in cell–cell communication and also reported to be a potential biomarker, and we attempted to link the findings concerning secreted miRNAs used in cell–cell communication tools and circulating miRNAs used as biomarkers. This discussion may increase broad interests and improve the current understanding of the importance of extracellular miRNAs in cell–cell communication. We would like to discuss about the vesicles, such as exosomes, microvesicles, and apoptotic bodies (Bobrie et al., 2011; Raposo and Stoorvogel, 2013). The mean size of exosomes, 40–100 nm in diameter, corresponds to that of the internal vesicles of multivesicular bodies from which they originate. Exosomes contain enriched amounts of some specific markers, especially those of endosomal origin including CD63, CD81, CD9, major histocompatibility complex class II, and so on. On the other hand, the size of microvesicles varies between 50 nm and 1 μm in diameter and the microvesicles are generated by budding at the plasma membrane toward the outside of the cell. However, the term of microvesicles has also been used for exosome-like vesicles and clear distinction of exosome and microvesicles has not been established;

therefore, we will use "extracellular vesicle (EV)" in this review, according to the definition of the International Society for Extracellular Vesicles, when describing studies using ultracentrifugation to isolate EVs.

miRNAs IN EXTRACELLULAR VESICLES OR NON-VESICLE ASSOCIATED miRNAs

It has been shown that EVs, such as exosomes, microvesicles, and apoptotic bodies, contain miRNAs with functions that have been previously reported (Valadi et al., 2007; Zernecke et al., 2009). The existence of non-vesicle associated miRNAs has also been reported. These miRNAs bind to HDL/LDL (Vickers et al., 2011) or RNA-binding proteins, such as Argonaute 2 (Ago2) (Arroyo et al., 2011; Turchinovich et al., 2011) and Ago1 (Turchinovich and Burwinkel, 2012). Interestingly, Arroyo et al. (2011) reported that circulating miRNAs in plasma are predominantly coupled with Ago2. The liver-specific miRNA, miR-122 has been detected only in protein-associated fractions, suggesting that hepatocytes might release miR-122 through a protein carrier pathway. In addition, Turchinovich and Burwinkel (2012) showed that not only Ago2 but also Ago1-bound miRNAs has been identified in human blood plasma. Intriguingly, they also found that some miRNAs in the plasma did not derive from blood cells under normal conditions. Although the abundance of miRNAs associated with RNA-binding proteins has been recognized, the functions of these miRNAs in cell–cell communications have not been clarified.

miR-210

miR-210 is a hypoxia-inducible miRNA that is activated by the master regulator of hypoxic stress, hypoxia-inducible factor (HIF)-1alpha in a variety of cell types (Chan et al., 2012). This miRNA has been implicated in erythropoiesis (Kosaka et al., 2008), iron homeostasis (Yoshioka et al., 2012), angiogenesis (Fasanaro et al., 2008), and cancer (Huang et al., 2009), which are also conditions associated with hypoxic stress. This miRNA has also been implicated in the regulation of DNA repair pathways (Crosby et al., 2009). The function of miR-210 has been investigated, although its exact contribution to the cancer microenvironment has not been determined.

Recently, we observed that EVs isolated from metastatic breast cancer cells promote metastasis via the induction of angiogenesis in the tumor (Kosaka et al., 2013). We also showed that EVs contain multiple angiogenic miRNAs, and one of them, miR-210, is responsible for angiogenesis. Indeed, the addition of miR-210-enriched EVs induced the activation of endothelial cells *in vitro* (Kosaka et al., 2013). Moreover, miR-210 expression is known to be inversely correlated with a disease-free and overall survival in breast cancer (Camps et al., 2008). Intriguingly, circulating miR-210 in breast cancer patients has been reported. The expression of circulating miR-210 is significantly higher in plasma from circulating tumor cell (CTC)-positive metastatic breast cancer patients compared with that in plasma from CTC-negative metastatic breast cancer patients and controls (Madhavan et al., 2012). The use of CTC as a prognostic marker in metastatic breast cancer has been well documented (Lianidou and Markou, 2011); however, adequate detection methods are still needed. Thus, circulating miRNAs could be used to predict the status of patients with metastatic breast cancer instead of detecting CTC. Moreover, the indication of CTC is associated with bad prognosis for cancer patients, and circulating miR-210 might contribute to this phenomenon (Madhavan et al., 2012).

Interestingly, circulating miR-210 levels were significantly higher in individuals with residual disease than in those who achieved a pathologically complete response to trastuzumab (Jung et al., 2012), administered at baseline before patients received neoadjuvant chemotherapy, as a part of the standard treatment for patients with human epidermal growth factor receptor 2 (HER-2)-positive breast cancer. Indeed, circulating miR-210 was derived from tumor cells, as reduced levels of circulating miR-210 were observed in the serum of patients after surgery compared with that in serum from patients before surgery. Furthermore, miR-210 expression was also higher in patients whose cancer metastasized to the lymph nodes. These results suggest that circulating miR-210 can be used to predict and perhaps monitor responses to therapies involving the use of trastuzumab. Elevated levels of HIF-1alpha were also associated with HER-2 over-expression in invasive breast cancer (Yamamoto et al., 2008). Moreover, the induction of HER-2 signaling in breast cancer cells increases HIF-1alpha protein and vascular endothelial growth factor (VEGF) mRNA expression (Laughner et al., 2001).

Taken together, these results suggest that miR-210 contributes to cancer development through immediate effects on the cancer cells and the modulation of the cancer cell microenvironment, and when secreted into peripheral blood, circulating miR-210 can be detected to predict the status of cancer cells in the tumor (**Table 1**).

EBV miRNAs

Epstein–Barr virus (EBV) encodes miRNAs, which were first reported viral miRNAs in human. A recent study on EBV-infected normal and neoplastic tissues revealed that distinct EBV miRNA expression profiles are produced in various latency programs, and EBV miRNAs play key roles in maintaining EBV persistence through the inhibition of apoptosis and the suppression of the host immune response (Forte and Luftig, 2011).

Previously, Pegtel et al. (2010) observed that functional EBV miRNAs, secreted from EBV-infected cells, are transferred to uninfected recipient cells. These authors showed the miRNA-mediated repression of confirmed EBV target genes, including *CXCL11*. Importantly, in a co-culturing system, containing EBV-transformed lymphoblastic B cells (donor cells) and primary immature monocyte-derived dendritic cells (recipient cells), approximately 2×10^3 copies of EBV-miRNA BART1-5p were detected in a subset of the recipient cells after 24 h, and this level increased fourfold (nearly 8×10^3 copies) after an additional 24 h of co-culture. Moreover, these authors confirmed that the expression of *CXCL11* in recipient cells was down-regulated within 24 h co-culture, suggesting that the transfer of 2×10^3 copies of EBV-miRNA is sufficient to suppress miRNA-target genes in recipient cells. Surprisingly, EBV miRNAs were present in both B cell and non-B cell fractions in peripheral blood mononuclear cells obtained from patients with an increased EBV load, although EBV DNA was restricted to the circulating B cell population. These observations indicated that viral miRNAs are functional in non-infected cells after the transfer of virus miRNAs from infected cells

Table 1 | The miR-210 studies in the cells and in the extracellular space.

Location	Phenotype	Origin of miR-210 expression	Reference
Intracellular	Anti-apoptosis in erythroid cells	Erythroid cells	Kosaka et al. (2008)
Intracellular	Regulate iron homeostasis by targeting ISCU and TfR1	Breast cancer cells	Yoshioka et al. (2012)
Intracellular	Regulate response to hypoxia by suppressing Ephrin-A3	Endothelial cells	Fasanaro et al. (2008)
Intracellular	Regulating the hypoxic response of tumor cells and tumor growth	Renal cancer cells	Huang et al. (2009)
Intracellular	Promote genetic instability via suppression of RAD52	Cervical carcinoma cells and breast cancer cells	Crosby et al. (2009)
Extracellular (endothelial cells)	Promote metastasis via the induction of angiogenesis through EVs delivery	Metastatic breast cancer cells	Kosaka et al. (2013)
Extracellular (blood)	High expression in serum from patients who have trastuzumab-resistance cancer	Drug resistance breast cancer cells	Jung et al. (2012)
Extracellular (blood)	High expression in CTC-positive patient	Breast cancer cells	Huang et al. (2009)

EVs, extracellular vesicles; ISCU, iron–sulfur cluster scaffold; TfR1, transferrin receptor 1; CTCs, circulating tumor cells; EPO, erythropoietin.

to non-infected cells through EVs. As shown above, this study provided the quantitative information on the level of extracellular miRNAs, which is essential for research on exosomal miRNA-mediated cell–cell communication. Information, such as the level of exosomal miRNAs required to suppress target molecules in recipient cells, might improve the quality of research on exosomal miRNAs in cell-cell communications.

Nasopharyngeal carcinoma (NPC) is a human epithelial malignancy associated with EBV, and EBV miRNAs are abundantly found in NPC tumors (Lo et al., 2012). Interestingly, viral miRNAs are secreted into the extracellular space from NPC cells with secreted EVs (Gourzones et al., 2010). In addition, these miRNAs are not only detected in plasma samples from NPC xenografted nude mice, but also in plasma samples from NPC patients. Moreover, EBV miRNAs were significantly up-regulated in tumor tissues compared with non-tumor biopsies, and the distinct presence of EBV miRNAs in the serum of NPC patients has been positively correlated with the cellular copy numbers of EBV miRNAs (Wong et al., 2012). Taken together, these results indicated that the viral miRNAs secreted from NPC cells, are contained inside EVs, resulting in the high stability for diffusion from the tumor site to the peripheral blood.

Interestingly, non-infected cells harbor miRNAs from viruses, and this fact might be an important aspect to reconsider infectious diseases. In the case of NPC, several studies have shown the contribution of EBV miRNAs to cancer development (Lo et al., 2012), and circulating miRNAs might be useful for the evaluation of patient status (Gourzones et al., 2010; Wong et al., 2012). Considering the delivery of EBV miRNAs through EVs, it is important to characterize the roles of EBV miRNAs in "non-infected cells" during the development of NPC. Moreover, miRNAs have been identified in numerous virus types, such as herpes B virus, human cytomegalovirus, herpes simplex virus, and Kaposi's sarcoma-associated herpes virus, among others. Thus, it would be important to examine the roles for these viral miRNAs in non-infected cells. This information might broaden the current understanding of infectious diseases caused by virus miRNAs.

miR-21

miR-21 is a well-characterized miRNA that contributes to the development of cancer (Schetter et al., 2008; Medina et al., 2010), and the target genes for miR-21 have been identified as well-known tumor suppressor genes, such as PTEN (Meng et al., 2007) and PDCD4 (Asangani et al., 2008). Thus, it is natural to examine the expression of circulating miR-21 in the serum of cancer patients for diagnosis. Indeed, several reports have shown the increased expression of circulating miR-21 in the serum of cancer patients, including diffuse large B cell lymphoma (DLBCL; Lawrie et al., 2008), osteosarcoma (Ouyang et al., 2013), colorectal cancer (Kanaan et al., 2012), hepatocellular carcinoma (HCC; Zhou et al., 2011), gastric cancer (Li et al., 2012), head and neck squamous cell carcinoma (Hsu et al., 2012), esophageal squamous cell carcinoma (Komatsu et al., 2012), prostate cancer (Yaman Agaoglu et al., 2011), and glioblastoma (Skog et al., 2008).

Skog et al. (2008) previously reported that glioblastoma tumor cells release EVs containing mRNA, miRNA, and angiogenic proteins, and these EVs are taken up by normal host cells, such as brain microvascular endothelial cells. These authors also showed that miR-21 levels are elevated in serum EVs from glioblastoma patients compared with controls. Circulating miR-21 has been reported in the serum/plasma obtained from various cancer patients, although the contribution of miRNAs to cancer development through EVs has not been discerned. miR-21 acts as an oncogenic miRNA in various cancer cells and also regulates various phenotypes in the cancer cell microenvironment. Indeed, miR-21 is not only involved in cancer development but also participates in homeostasis (Niu et al., 2011); thus, understanding the contribution of miR-21 to the cellular microenvironment will increase the global understanding of animal development.

miR-21, associated with RNA-binding proteins, has also been detected in the culture supernatant from breast cancer cell

lines (Turchinovich et al., 2011) and serum from healthy donors (Arroyo et al., 2011), and the abundance of miR-21 in the extracellular space has been recognized as shown above. Thus, determining the biological significance for miR-21 binding to Ago2 might provide a better understanding of miRNA-associated cell–cell communication in cancer development.

miR-126

One of the earliest studies to show the transfer of miRNAs between the cells was revealed by the study of apoptotic bodies. In this study, the authors found that endothelial cell-derived apoptotic bodies contained miR-126 and these apoptotic bodies convey paracrine alarm signals to recipient vascular cells during atherosclerosis (Zernecke et al., 2009). In addition, another study also showed that secretory miR-126 was precipitated in the angiogenesis. The EVs from $CD34^+$ peripheral blood mononuclear cells exhibited proangiogenic properties via the transfer of miR-126 (Mocharla et al., 2013). Cantaluppi et al. (2012a) reported that EVs released from endothelial progenitor cells (EPCs) enhanced islet endothelial cell proliferation, migration, anti-apoptosis, and organization in vessel-like structures. They also found that EVs from EPCs contained the miR-126 and miR-296 and that these miRNAs contributed to the angiogenesis properties, suggesting that EVs from EPCs activate an angiogenic program in islet endothelium (Cantaluppi et al., 2012a). They also reported that miR-126 in EVs from EPCs contributed to the prevention of the ischemic acute injury in kidney by enhanced tubular cell proliferation, reduced apoptosis, and leukocyte infiltration (Cantaluppi et al., 2012b). In addition, EPC-derived EVs were able to induce neoangiogenesis and to enhance recovery in a hindlimb ischemia (Ranghino et al., 2012).

Although circulating miR-126 was enriched in systemic lupus erythematosus (Wang et al., 2012a), expression of circulating miR-126 was decreased in the breast cancer (Wang et al., 2010) and malignant mesothelioma (Tomasetti et al., 2012). Whereas there are only a few reports regarding the circulating miR-126, secretory miR-126 from cells has a great activity of endothelial cells activations as shown in above. Therefore, it is tempting to investigate the potential of miR-126 as biomarker in diseases which were caused by the abnormal angiogenesis.

miR-451

Kogure et al. (2011) showed a subset highly enriched miRNAs within EVs from HCC cells and identified a target of these miRNAs, transforming growth factor β activated kinase-1. Indeed, loss of this pathway resulted in the enhancement of transformed cell growth in recipient cells. One of the miRNAs that they identified in this study, miR-451, was found in the serum from patient with liver disease. Murakami et al. (2012) investigated the disease parameters in patients with chronic hepatitis C (CHC) by focusing on miRNAs isolated from EV-enriched fraction in serum. They successfully classified CHC and normal liver with 96.59% accuracy using the expression patterns of nine miRNAs including miR-451 (Murakami et al., 2012).

miR-223

Ismail et al. (2013) found that EVs from macrophage contained miR-223, and that this miR-223 was transported to target cells, including monocytes, endothelial cells, epithelial cells, and fibroblasts, and was functionally active. Macrophages are found in all tissues and they play roles in development, homeostasis, tissue repair, and immunity, and thus are therapeutic targets in many human diseases (Wynn et al., 2013). Indeed, an increased level of circulating miR-223 was found in serum/plasma from patients with gastric cancer (Li et al., 2012), non-small cell lung carcinoma (Sanfiorenzo et al., 2013), hepatitis B virus-related HCC (Zhou et al., 2011), NPC (Zeng et al., 2012), hypertension-induced heart failure (Dickinson et al., 2013), systemic lupus erythematosus, rheumatoid arthritis (Wang et al., 2012a), sepsis (Wang et al., 2012b), ischemic injury (Yu et al., 2009), and osteoarthritis (Okuhara et al., 2012). To date, origins of this circulating miR-223 have not been investigated yet; however, from the reports shown above, macrophage is probable candidate of origin for circulating miR-223. Interestingly, miR-223 is found not only in EVs but also in HDL (Vickers et al., 2011). In addition, miR-223 concentration in HDL was increased 3,780-fold with familial hypercholesterolemia when compared with controls. The HDL is involved in the transport of cholesterol from lipid-enriched macrophages of atherosclerotic arteries to the liver. Recently, Wagner et al. (2013) reported that miR-223 was detected at concentrations >10,000 copies/μg in HDL from healthy subjects. However, HDL-bound miR-223 contributed to only 8% of the total circulating miRNAs. In addition, a significant uptake of HDL-bound miRNAs into endothelial cells, smooth muscle cells, or peripheral blood mononuclear cells was not observed, suggesting that the lipoprotein-associated miR-223 does not regulate the function of the studied cells *in vitro*. Knowing the function of secretory miR-223 in macrophage homeostasis *in vivo* might lead to the development of not only the disease biomarker, but also the novel therapy against atherosclerosis.

miR-150

Zhang et al. (2010) demonstrated that miR-150 from monocytic cells were delivered into endothelial cell, and this miR-150 reduced its target gene, c-Myb, expression in endothelial cells, resulting in the enhancement of cell migration in endothelial cell both *in vitro* and *in vivo*. They also found that monocyte-secreted miR-150 promoted angiogenesis *in vivo* using tumor-implanted mice and ob/ob mice as models (Li et al., 2013). Intriguingly, the expression of miR-150 was higher in EVs isolated from the plasma of patients with atherosclerosis, and these EVs promoted endothelial cell migration compared to EVs from healthy donors (Zhang et al., 2010). A high level of circulating miR-150 was reported in several diseases including idiopathic childhood nephrotic syndrome (Luo et al., 2013), acute myeloid leukemia (Fayyad-Kazan et al., 2013), and so on. On the contrary, miR-150 serum concentrations upon admission were closely associated with intensive care unit (ICU) survival as well as long-term survival, and low miR-150 levels indicated an unfavorable prognosis (Roderburg et al., 2013).

SUMMARY AND PERSPECTIVES

In this review, we presented the results obtained from research on miRNAs to provide a better understanding of the relationship

between secreted miRNAs which contribute to cell–cell communication in cancer development, and circulating miRNAs which are used as disease biomarkers.

Recently, a novel concept for biomarkers, called "liquid biopsy," has been proposed (Forshew et al., 2012; Murtaza et al., 2013). Liquid biopsy would be useful for numerous diagnostic applications and avoid the need for tumor tissue biopsies. Current studies have shown that genomic alterations in solid cancer can be characterized through the massively parallel sequencing of circulating cell-free tumor DNA released from cancer cells into the plasma (Forshew et al., 2012; Murtaza et al., 2013). This suggests that circulating miRNAs are also good candidates for liquid biopsy, as the quantities and sequences of miRNAs convey information for diagnosis. Particularly, circulating miRNAs, which have been previously shown to function in cell–cell communication, might be good candidates for this application. Therefore, we emphasize that it is important to investigate the function of secretory miRNAs in cell–cell communication, and in parallel explore the usefulness of these molecules as biomarkers using animal models.

Much of the current research on circulating miRNAs for disease biomarkers does not describe the types of circulating miRNAs, such as EVs, microvesicles, HDL/LDLs, or RNA-binding proteins that are present in human body fluids. As previously discussed, focusing on a specific type of circulating miRNAs, such as exosomal miRNAs or miRNAs bound to RNA-binding proteins, might be useful as disease biomarkers compared with analyzing the total miRNA in human body fluids. Indeed, EV-enriched fractions isolated from patients with liver disease were useful for the determination of disease progression compared with the profiles obtained using total miRNA present in serum samples (Murakami et al., 2012). Therefore, it is essential that future studies concerning circulating miRNAs for diagnostic purposes should focus on the type of circulating miRNAs present in body fluids.

One of the crucial issues in research on cell–cell communication by secretory miRNAs is whether the secretory miRNAs which researcher identified are really physiologically functional enough or not. This issue might be revealed by showing the quantitative data of secretory miRNAs in more detail, such as the number of EVs, the number of miRNAs, and the number of cells. In addition, in the case of functional demonstration of secretory miRNAs, over-expression or knock-down of secretory miRNAs was performed; however, contamination of exogenous miRNAs, such as synthetic miRNAs, should be cared since the amount of those exogenous miRNAs are usually introduced in excess. The study on extracellular miRNAs has just begun. Thus, the researcher working on the EVs needs to take care of the physiological amount of those molecules in their research field.

Another crucial issue of extracellular miRNAs that how these miRNAs are secreted from cells and how these miRNAs work in the cells has not been answered yet, although recent reports proved the physiological and pathological importance of secretory miRNAs not only *in vitro* but also *in vivo*. We previously found that secretion of miRNAs from cells was regulated by neutral sphingomyelinase 2, which is known as a rate-limiting enzyme of ceramide biosynthesis and triggers secretion of EVs (Kosaka et al., 2010b). Although the molecules that are essential for EVs secretion has been reported, their contribution to miRNAs secretion has not been tested yet. One of the most important points for understanding of miRNAs secretion is the identification of a protein that binds to miRNAs in EVs. miRNAs are strongly bound to the Ago2 protein, which is a main component of the RNA-induced silencing complex (RISC), in the cells (Kim et al., 2009), but this molecule is not found in EVs (Gibbings et al., 2009). Meanwhile, knockdown of GW182, another main component of the RISC, reduced miRNA secretion via EVs. Interestingly, however, GW182 was not detected in the EVs from HEK293 (Yao et al., 2012). In contrast to the above report, GW182 can be found in EVs from monocyte, HeLa cells and *ex vivo*-derived dendritic cells (Gibbings et al., 2009). These paradoxical observations indicate that further experiments are required to elucidate whether there is a role for GW182 in miRNA secretion. Identification of proteins that are responsible for the transport of miRNAs from inner cells to inner EVs might reveal many of mysteries of secretory miRNAs in cell–cell communications.

Circulating RNA has been previously considered as "trash" from cells; however, we propose that this "trash" serves as a communication tool and should therefore be referred to as "treasure." Analyzing circulating miRNAs in human body fluids might provide a method for "listening" to the communication between cells, leading to the development of disease treatments based on the mechanisms of secreted miRNAs in cancer development.

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Systemically Injected Exosomes Targeted to EGFR Deliver Antitumor MicroRNA to Breast Cancer Cells

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Despite the therapeutic potential of nucleic acid drugs, their clinical application has been limited in part by a lack of appropriate delivery systems. Exosomes or microvesicles are small endosomally derived vesicles that are secreted by a variety of cell types and tissues. Here, we show that exosomes can efficiently deliver microRNA (miRNA) to epidermal growth factor receptor (EGFR)-expressing breast cancer cells. Targeting was achieved by engineering the donor cells to express the transmembrane domain of platelet-derived growth factor receptor fused to the GE11 peptide. Intravenously injected exosomes delivered let-7a miRNA to EGFR-expressing xenograft breast cancer tissue in RAG2$^{-/-}$ mice. Our results suggest that exosomes can be used therapeutically to target EGFR-expressing cancerous tissues with nucleic acid drugs.

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INTRODUCTION

MicroRNAs (miRNAs) are small (20–22 nucleotides) noncoding RNA molecules that bind to partially complementary mRNA sequences, resulting in target degradation or translation inhibition.[1] A growing pool of evidence suggests that miRNA-related gain- or loss-of-function mutations can cause the development and/or progression of cancer.[2] For example, let-7a is thought to be a tumor suppressor that inhibits the malignant growth of cancer cells by reducing RAS and HMGA2 expression. Reduced expression levels of let-7 have been observed in colon, lung, ovary, and breast cancer cells.[3] Therefore, miRNA replacement therapies have emerged as promising treatment strategies for malignant neoplasms. Yet although miRNA-based modalities may eventually prove effective, their clinical application has been hampered by a lack of appropriate delivery systems.

Exosomes or microvesicles are small vesicles (50–100 nm in diameter) that are secreted by a variety of cell types and tissues.[4] Of clinical interest, tumor cells have been shown to release exosomes containing miRNA[5] and miRNAs secreted from donor cells can be taken up and function in recipient cells.[6,7] These data indicate that exosomes are natural carriers of miRNA that could be exploited as an RNA drug delivery system. For instance, Alvarez-Erviti *et al.* recently used exosomes with modified membranes containing a neuron-specific peptide to deliver small-interfering RNA (siRNA) to mouse brain tissue.[8] Nevertheless, the utility of exosomes as carriers of cancer therapies remains largely unknown.

A number of human tumors of epithelial origin display elevated epidermal growth factor receptor (EGFR) expression, suggesting that EGFR could serve as a receptor target in cancer drug delivery systems.[9] Because the EGFR ligand epidermal growth factor (EGF) is strongly mitogenic and neoangiogenic, however, an alternative ligand is needed for clinical applications.

The GE11 peptide (amino-acid sequence YHWYGYTPQNVI) binds specifically to EGFR, but is markedly less mitogenic than EGF.[10] Additionally, GE11-conjugated polyethylenimine vectors and polyethylene glycol–conjugated liposomes have been shown to be less mitogenic, and can efficiently transfect genes into cells expressing high levels of EGFR or tumor xenografts.[10–13] These studies indicated that the GE11 peptide is likely superior to EGF for clinically targeting EGFR-expressing tumors.

In this study, we examined exosomes as drug delivery carriers in a model of cancer. Modified exosomes with the GE11 peptide or EGF on their surfaces delivered miRNA to EGFR-expressing cancer tissues; intravenously injected exosomes targeting EGFR delivered let-7a specifically to xenograft breast cancer cells in RAG2$^{-/-}$ mice. These data indicate that exosomes targeted to EGFR-expressing cells may provide a platform for miRNA replacement therapies in the treatment of various cancers.

RESULTS

GE11- and EGF-positive exosomes

GE11 peptide specifically binds to EGFR, but is less mitogenic than EGF.[10] To generate GE11- or EGF-positive exosomes, sequence encoding GE11 or EGF was cloned into the pDisplay vector. This vector promotes the expression of proteins on plasma membranes

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