3.2. Construction of Luciferase Reporter Plasmids

DNA fragments containing the 3'-UTRs of TET1, TDG, DNMT3A and DNMT3B were amplified by PCR using primers containing XbaI or FseI restriction sites. Amplified fragments were cloned into the corresponding sites in the 3'-UTR of the luciferase reporter vector, pGL3-Control (Promega, Madison, WI, USA). The primer sequences were as follows: TET1 sense, GCT CTA GAG CCC TAT AAC CAT TGG GTC T; TET1 antisense, GGG GCC GGC CTG AAG CAG CTG AAG CAA TAA AC; TDG sense, GCT CTA GAC AGC CCC ATA AGA TTC CAG A; TDG antisense, GGG GCC GGC CTG ATG CAA GGC ACT TCA AA; DNMT3A sense, GCT CTA GAC GAA AAG GGT TGG ACA TCA T; DNMT3A antisense, GGG GCC GGC CGC CGA GGG AGT CTC CTT TTA; DNMT3B sense, GCT CTA GAC TGA CTC TTG CAG GGG TAG C; and DNMT3B antisense, GGG GCC GGC CGT TAC GTC GTG GCT CCA GTT.

3.3. Reporter Assay

Luciferase reporter plasmids were transiently transfected into A548 and PC9 cells using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA), according to the manufacturer's protocol. Briefly, cells seeded into a 24-well tissue culture dish were exposed to transfection mixtures containing 0.1 μg of luciferase reporter plasmid, 0.05 μg of pRL-TK control vector (Promega) and 10 pmol of miRNA. Cells were harvested 48 h after transfection. Luciferase assays were performed according to the manufacturer's protocol (Promega). The pRL-TK plasmid was used to normalize firefly luciferase activity to Renilla luciferase activity, to correct for transfection efficiency. Control miRNA and miR-29 were purchased from B-Bridge (Tokyo, Japan).

3.4. Transfection of Cells with miRNA

Transient transfections of A548 and PC9 cells with miRNAs were performed using Lipofectamine 2000 (Invitrogen), according to the protocol recommended by the manufacturer. Briefly, cells seeded into a 6-well tissue culture dish were transfected with 100 pmol of miRNA. Cells were harvested 48 h after transfection. Transfected miRNAs are mature miRNA mimic molecules. DNA and RNA species were extracted and were subjected to LUMA and quantitative RT-PCR assays, respectively. The primer sequences used for quantitative RT-PCR were as follows: TET1 sense, CCG AAT CAA GCG GAA GAA TA; TET1 antisense, TAA AAT GGG GTT CGG TTT CA; TDG sense, AGG AGC TTC AGC CAT CAG TT; TDG antisense, GAA TGG AAG CGG AGA ACG; DNMT3A sense, ATA AGC TGG AGC TGC AGG AG; DNMT3A antisense, TGA AGA CAG GAA AAT GCT GGT; DNMT3B sense, ATG AAG GTT GGC GAC AAG AG; and DNMT3B antisense, CCC TGT GAG CAG CAG AAA CT; ACTB sense, GAT GCA GAA GGA GAT CAC TGC; and ACTB antisense, GTA CTT GCG CTC AGG AGG AG.

3.5. LUMA Assay

LUMA assays were performed as described previously [21]. Briefly, genomic DNA (300–500 ng) was cleaved with HpaII (45 U) and EcoRI (25 U) or MspI (45 U) and EcoRI (22.5 U) in two separate

15 μL reactions containing 33 mM Tris-acetate (pH 7.9), 10 mM Mg-acetate, 66 mM K-acetate and 0.1 mg/mL BSA. The reactions were incubated at 37 °C for 4 h, and then, 15 μL of annealing buffer (20 mM Tris-acetate (pH 7.6) and 2 mM Mg-acetate) was added. Samples were placed in a PyroMark 24 pyrosequencing system (Qiagen, Valencia, CA, USA), and the instrument was programmed to add dNTPs in four consecutive steps: Step 1, dATP (the derivative dATPαS was used, because it does not react directly with luciferase and, hence, prevents the generation of non-specific signals); Step 2, mixture of dGTP and dCTP; Step 3, dTTP; and Step 4, mixture of dGTP and dCTP. Peak heights were calculated using the PyroMark 24 software. The HpaII/EcoRI and MspI/EcoRI ratios were calculated as (dGTP + dCTP)/dATP. The HpaII/MspI ratio was defined as (HpaII/EcoR1)/(MspI/EcoRI). The global methylation ratio was calculated as one minus the HpaII/MspI ratio.

3.6. Quantitative Methylation-Specific PCR

Quantitative PCR was performed using bisulfite-converted genomic DNA. Primers specific to methylated RASSF1 DNA were as follows: RASSF1 sense, TTA GCG TTT AAA GTT AGC GAA GTA C; and RASSF1 antisense, ATA AAC TCA AAC TCC CCC GAC.

4. Conclusions

Members of the miR-29 family repress the activities of both DNA methyltransferases and DNA demethylases, which have opposing functions in the control of DNA methylation. The results presented in this study demonstrate that the miR-29 family directly represses DNA methyltransferases, as well as TET1 and TDG, which are two major factors involved in DNA demethylation. These findings suggest that miR-29 suppresses tumorigenesis by protecting against changes in the existing DNA methylation status, rather than by preventing *de novo* methylation.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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Competitive Interactions of Cancer Cells and Normal Cells via Secretory MicroRNAs*[S]

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Background: Homeostatic cell competitive system between cancerous cells and non-cancerous cells is considered as the reason for tumor initiation.
Results: Exosomal tumor-suppressive microRNAs secreted by non-cancerous cells inhibit the proliferation of cancerous cells.
Conclusion: Exosomal tumor-suppressive microRNAs act as an inhibitory signal for cancer cells in a cell-competitive process.
Significance: This provides a novel insight into a tumor initiation mechanism.

Normal epithelial cells regulate the secretion of autocrine and paracrine factors that prevent aberrant growth of neighboring cells, leading to healthy development and normal metabolism. One reason for tumor initiation is considered to be a failure of this homeostatic cell competitive system. Here we identify tumor-suppressive microRNAs (miRNAs) secreted by normal cells as anti-proliferative signal entities. Culture supernatant of normal epithelial prostate PNT-2 cells attenuated proliferation of PC-3M-luc cells, prostate cancer cells. Global analysis of miRNA expression signature revealed that a variety of tumor-suppressive miRNAs are released from PNT-2 cells. Of these miRNAs, secretory miR-143 could induce growth inhibition exclusively in cancer cells *in vitro* and *in vivo*. These results suggest that secretory tumor-suppressive miRNAs can act as a death signal in a cell competitive process. This study provides a novel insight into a tumor initiation mechanism.

Competitive interactions among cells are the basis of many homeostatic processes in biology. In *Drosophila*, normal epithelial cells compete with transformed ones for individual survival, which is a process called cell competition (1, 2). If a given group of cells was exposed to some stress, it would be separated into subpopulations of cells with different levels of damage. In noncompetitive conditions, cells with severe damage die in a short time, whereas moderately damaged cells survive to the next generation, indicative of the transduction of a negative phenotype. On the other hand, in competitive conditions even slightly damaged cells are eliminated from the cell group because healthy cells, the "winners," convey death signals to damaged cells, the "losers," and the losers reciprocally confer growth signals to the winners. This feed-forward regulation enables the cell population to eradicate abnormal cells and maintain the same number of normal cells in a limited niche.

Oncogenesis is characterized by genetic and metabolic changes reprogramming living cells to undergo uncontrolled proliferation (3). This suggests that the abnormal cells that are originally destined for elimination can survive and expand against the cell competitive regulation, leading to the formation of a tumor mass. Consistently with this concept, Bondar and Medzhitov (4) showed that the cell competition process involves p53, a tumor-suppressive gene, between the hematopoietic stem cells and progenitor cells, suggesting that gene modifications of p53 could disturb the homeostatic mechanism and give rise to tumor initiation. It is conceivable that p53 target genes could be associated with intercellular communication between winners and losers; however, this literature has not answered the question of whether this regulatory system is mediated by contact-dependent or contact-independent manner. More than 10 years ago a pioneer study suggested that non-cancerous cells co-cultured with cancer cells inhibit the growth of cancer cells *in vitro* (5). This result indicated that humoral factors could be involved in cell competition as intercellular communicators (6).

As recently as a few years ago it was believed that RNAs could not behave as extracellular signal molecules because of their vulnerability to the attack of ribonucleases largely existing in body fluid. Evidence is presently increasing to show that miRNAs[4] contained in exosomes are released from mammalian

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[4] The abbreviations used are: miRNA, microRNA; CM, conditioned medium; luc, luciferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; QRT-PCR, quantitative real time PCR.

cells and act as a signal transducer (7). It is important that many different tumor-suppressive miRNAs, such as miR-16 and miR-143, are down-regulated in cancer cells, resulting in tumorigenesis, tumor progression, and metastasis (8–11). Taken together, these findings suggest that secretory miRNAs may have favorable aspects for anti-proliferative signals mediating cell competition.

In this report we show that miR-143 expression in normal prostate cells, PNT-2 cells, is higher than that in prostate cancer cells, PC-3M-luc cells, and that miR-143 released from non-cancerous cells transfers growth-inhibitory signals to cancerous cells *in vitro* and *in vivo*. These results suggest that secretory tumor-suppressive miRNAs might be a death signal from winners to losers in the context of cell competition. Secretory miRNAs can be conducive to the maintenance of normal growth and development.

EXPERIMENTAL PROCEDURES

Reagents—Mouse monoclonal anti-KRAS (F234) (sc-30) was purchased from Santa Cruz. Rabbit polyclonal anti-ERK5 (#3372) was purchased from Cell Signaling. Mouse monoclonal anti-actin, clone C4 (MAB1501), was obtained from Millipore. Mouse monoclonal ant-human-CD63 antibody (556019) was purchased from BD Pharmingen. Peroxidase-labeled anti-mouse and anti-rabbit antibodies were included in the Amersham Biosciences ECL PLUS Western blotting Reagents Pack (RPN2124) (GE Healthcare). Synthetic *Caenorhabditis elegans* miRNA cel-miR-39 was synthesized by Qiagen (Valencia, CA). Synthetic hsa-miR-143 (pre-miR-143), the negative control 1 (NC1), has-miR-143 inhibitor molecule (anti-miR-143), and the negative control inhibitor molecule (anti-NC) were purchased from Ambion (Austin, TX). GW4869 was purchased from Calbiochem. Geneticin was purchased from Invitrogen.

Cell Culture—PNT-2 cells, immortalized normal adult prostatic epithelial cell line, were purchased from the DS Pharma Biomedical Co., Ltd. (Osaka, Japan). HEK293 cells, a human embryonic kidney cell line (CRL-1573), were obtained from American Type Culture Collection (Manassas, VA). HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (FBS) and an antibiotic-antimycotic (Invitrogen) at 37 °C in 5% CO_2. PNT-2 and the prostate cancer cell line, PC-3M-luc cells, continuously expressing firefly luciferase (Xenogen, Alameda, CA), were cultured in RPMI containing 10% heat-inactivated FBS and an antibiotic-antimycotic at 37 °C in 5% CO_2.

Preparation of Conditioned Medium and Exosomes—Before the collection of culture medium, cells were washed 3 times with Advanced RPMI containing an antibiotic-antimycotic and 2 mM L-glutamine (medium A), and the medium was switched to fresh medium A. After incubation for 3 days, medium A was collected and centrifuged at 2000 × *g* for 10 min at room temperature. To thoroughly remove cellular debris, the supernatant was centrifuged again at 12,000 × *g* for 30 min at room temperature or filtered through a 0.22-μm filter (Millipore). The conditioned medium (CM) was then used for miRNA extraction and functional assays as well as exosome isolation.

For exosome preparation the CM was ultracentrifuged at 110,000 × *g* for 70 min at 4 °C. The pellets were washed with 11 ml of PBS, and after ultracentrifugation they were resuspended in PBS. The exosome fraction was measured for its protein content using the Micro BCA Protein Assay kit (Thermo Scientific, Wilmington, DE).

Isolation of MicroRNAs—Isolation of extracellular and cellular miRNAs was performed using the miRNeasy Mini Kit (Qiagen). Two hundred microliters of conditioned medium or cell lysate was diluted with 1 ml of Qiazol Solution. After 5 min of incubation, 10 μl of 0.1 nM cel-miR-39 was added to each aliquot followed by vortexing for 30 s. Subsequent extraction and filter cartridge work were carried out according to the manufacturer's protocol.

Quantitative Real Time PCR (QRT-PCR)—The method for QRT-PCR has been previously described (7). 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 Biosystems. Cel-miR-39 and RNU6 were used as an invariant control for the CM and cells, respectively.

Immunoblot Analysis—SDS-PAGE gels, SuperSep Ace 5–20% (194–15021) (Wako), were calibrated with Precision Plus Protein Standards (161–0375) (Bio-Rad), and anti-KRAS (1:100), anti-ERK5 (1:1000), anti-CD63 (1:200), and anti-actin (1:1000) were used as primary antibodies. The dilution ratio of each antibody is indicated in parentheses. Two secondary antibodies (peroxidase-labeled anti-mouse and anti-rabbit antibodies) were used at a dilution of 1:10,000. Bound antibodies were visualized by chemiluminescence using the ECL PLUS Western blotting detection System (RPN2132) (GE Healthcare), and luminescent images were analyzed by a LuminoImager (LAS-3000; Fuji Film, Inc.). Only gels for CD63 (BD Biosciences) detection were run under non-reducing conditions.

Plasmids—The primary-miR-143 expression vector was purchased from TaKaRa BIO. For luciferase-based reporter gene assays, pLucNeo was constructed by inserting a firefly luciferase gene derived from the pGL3-control (Promega) into the pEYFP-1 vector (Clontech) at BglII and AflII sites. The sensor vector for miR-143 was constructed by introducing tandem binding sites with perfect complementarity to miR-143 separated by a four-nucleotide spacer into the NotI site of psiCHECK2 (Promega). The sequences of the binding site are as follows: 5′-AAACCTAGAGCGGCCGCGAGCTACAGTGCT*TCATCTC*AAAGAATTCTTGAGCTACAGTGCT*TCATCTC*AGCGGCCGCTGGCCGCAA-3′ (sense) and 5′-TTGCGGCCAGCGGCCGCTGAGATGAAGCACTGTAGCTCAAGAATTCTTTGAGATGAAGCACTGTAGCTCGCGGCCGCTCTAGGTTT-3′ (antisense). The "seed" sequence of miR-143 is indicated by bold italics. In a mutated miR-143 sensor vector, the seed sequence, TCATCTC, was displaced with GACGAGA. All the plasmids were verified by DNA sequencing.

Transient Transfection Assays—Transfections of 10 nM miR-143 mimic and 3 nM anti-miR-143 were accomplished with the DharmaFECT Transfection Reagent (Thermo Scientific) according to the manufacturer's protocol. The total amounts of miRNAs for each transfection were equally adjusted by the addition of NC1 and anti-NC, respectively.

Establishment of Stable Cell Lines—Stable HEK293 cell lines that express miR-143 were generated by selection with 300 μg/ml Geneticin. HEK293 cells were transfected with 0.5 μg of the pri-miR-143 expression vector at 90% confluency in 24-well dishes using a Lipofectamine LTX reagent in accordance with the manufacturer's instructions. Twelve hours after the transfection, the cells were re-plated in a 10-cm dish followed by a 3-week selection with the antibiotic. Ten surviving single colonies were picked up from each transfectant and then cultured for another 2 weeks. The cells expressing the largest amount of miR-143 among transfectants were used as miR-143 stably expressing cells.

Luciferase Reporter Assay—HEK293 cells were cultured at a density of 1×10^4 cells/well in 96-well tissue culture plates overnight, and miRNA transfections or the addition of CM was performed. The cells were harvested, and renilla luciferase activity was measured and normalized by firefly luciferase activity (10). All assays were performed in triplicate and repeated at least three times, and the most representative results are shown.

Cell Growth Assay—PC-3M-luc cells were seeded at a density of 2×10^3 cells/well in a 96-well plate. The following day the cells were transfected with mature miRNAs or incubated with a CM. Twenty-four hours later the culture medium of the transfected cells was switched to medium A, whereas the conditioned medium was not changed. After a 3-day culture, cells were harvested for the measurement of firefly luciferase activity. To know the cellular proliferation by the tetrazolium-based colorimetric MTT assay, 20 μl CM of TetraColor ONE (SEIKAGAKU Corp., Tokyo, Japan) was added to each well after 72 h of culture. After 2–4 h of incubation at 37 °C, the optical density was measured at a wavelength of 450 nm using a microplate reader.

PKH67-labeled Exosome Transfer—Purified exosomes derived from PNT-2 CM were labeled with a PKH67 green fluorescent labeling kit (Sigma). Exosomes were incubated with 2 μM PKH67 for 5 min, washed 4 times using a 100-kDa filter (Microcon YM-100, Millipore) to remove excess dye, and incubated with PC-3M-luc cells at 37 °C.

Co-culture Experiment—In co-culture experiments, 2×10^5 cells/well of PNT-2 cells were plated in 6-well plates. To stain the PNT-2 cells with BODIPY-TR-ceramide (Invitrogen), 5 μM BODIPY-TR-ceramide in a non-serum culture medium was added and incubated with the cells at 37 °C. After 30 min the cells were rinsed several times with a non-serum culture medium and incubated in a fresh medium at 37 °C for an additional 30 min. After the staining of PNT-2 cells by BODIPY-TR-ceramide, labeling of PC-3M-luc cells with PKH67 was performed in accordance with the manufacturer's instructions. After that, labeled PC-3M-luc cells were added and co-cultured with PNT-2 cells for 12 h at 37 °C.

Microarray Analysis—To detect the miRNAs in exosomes and cells derived from PNT-2 and PC-3M-luc cells, 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.

Evaluation of Tumor-suppressive miRNA Delivery to Subcutaneously Implanted Prostate Cancer Cell Line in Mice—Animal experiments in this study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. Seven-week-old male Balb/c athymic nude mice (CLEA Japan, Shizuoka, Japan) were anesthetized by exposure to 3% isoflurane for injections and *in vivo* imaging. Four days ahead of the first CM injection, the anesthetized animals were subcutaneously injected with 5×10^5 PC-3M-luc cells suspended in 100 μl of sterile Dulbecco's phosphate-buffered saline into each dorsal region. Five hundred μl of CM derived from miR-143-overexpressing HEK293 cells and control cells were daily injected into each tumor from day 0 to 6. 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 2.50 software (Xenogen).

RESULTS

Suppression of Prostate Cancer Cell Proliferation by Conditioned Medium Isolated from Non-cancerous Prostatic Cell—Cell competition is a homeostatic mechanism for the accommodation of an appropriate number of cells in a limited niche or stroma (1). Based on this idea it is possible that the cell competition between normal and abnormal cells frequently occurs in a precancerous state. Of note is that non-cancerous cells suppress cancer cell development by contact-independent interaction (12). For instance, endothelial cells provide the major extracellular heparan sulfate proteoglycan as anti-proliferative signals (12); however, the molecular mechanism by which the other types of cells in a tumor environment associate with cancer cells is not fully understood.

To analyze the mechanism, we treated a hormone-insensitive prostatic carcinoma cell line, PC-3M-luc cells, with a CM from the non-cancerous prostate cell line PNT-2 cells. After a 3-day incubation, the PNT-2 CM inhibited the growth of the PC-3M-luc cells up to ~10% compared with the cell growth treated by fresh culture medium (Fig. 1*A*; compare *lanes 1* and *3*). In contrast, the growth of PC-3M-luc cells incubated in the CM of PC-3M-luc cells themselves showed no inhibitory effect (Fig. 1*A*; compare *lanes 1* and *2*). To determine that the performed treatments did not affect the luciferase activity, we also used the colorimetric MTT assay to measure the cell growth of PC-3M-luc cells. As shown in supplemental Fig. 1*A*, not only luciferase assay but also MTT assay show the inhibition of PC-3M-luc cell proliferation by the addition of PNT-2 cells derived CM, indicating that our treatment did not affect the luciferase activity. These results indicate that the non-cancerous cells may secrete some molecules that can suppress cancer cell proliferation.

In a recent report we showed that miRNAs contained in exosomes are secreted and that their secretion is tightly regulated by neutral sphingomyelinase 2, which is known to hydrolyze sphingomyelins to generate ceramides and trigger the budding

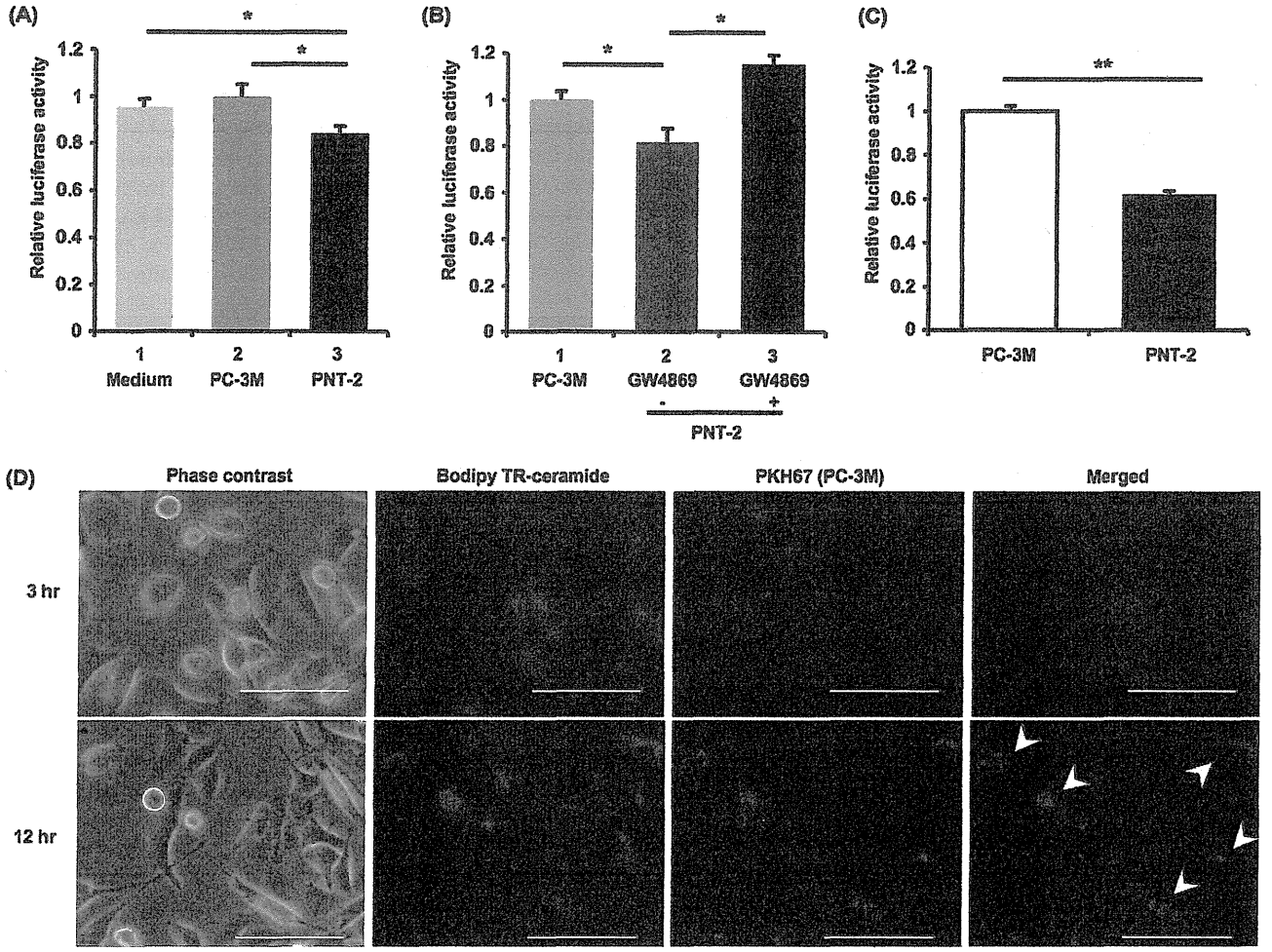

FIGURE 1. Suppression of cancerous cell proliferation by exosome isolated from non-cancerous cells. *A*, cell growth inhibition by a conditioned medium derived from PNT-2 cells is shown. PC-3M-luc cells were incubated for 3 days in a conditioned medium isolated from PC-3M-luc cells, PNT-2 cells, or a culture medium followed by a cell growth assay as described under "Experimental Procedures." The values on the *y* axis are depicted relative to the normalized luciferase activity of culture medium-treated cells, which is defined as 1. Each *bar* is presented as the mean S.E. ($n = 3$). *, $p < 0.05$ as compared with culture medium-treated PC-3M-luc cells; Student's *t* test. *B*, treatment with GW4869 to donor cells restored the reduced cell growth by the PNT-2-derived CM is shown. Donor PNT-2 cells were incubated in the presence or absence of 10 μM GW4869 for 2 days. The conditioned medium from PC-3M-luc cells was used as a control. The values on the *y* axis are depicted relative to the normalized luciferase activity of PC-3M-luc-conditioned medium-treated cells, which is defined as 1. Each *bar* is presented as the mean S.E. ($n = 3$). *, $p < 0.05$; Student's *t* test. *C*, cell growth inhibition by exosomes derived from PNT-2 cells is shown. PC-3M-luc cells were incubated in exosomes isolated from PNT-2 cells or PC-3M-luc cells followed by a cell growth assay, as described under "Experimental Procedures." The values on the *y* axis are depicted relative to the normalized luciferase activity of cells treated with exosomes derived from PC-3M-luc cells is defined as 1. Each *bar* is presented as the mean S.E. ($n = 3$). **, $p < 0.005$, as compared with exosomes isolated from PC-3M-luc cells; Student's *t* test. *D*, shown are fluorescent photos of BODIPY-ceramide-labeled PNT-2 and PC-3M-luc cells marked by PKH67. PNT-2 cells and PC-3M-luc cells were labeled with red fluorescent BODIPY-ceramide and green fluorescent PKH67, respectively, as described under "Experimental Procedures." After treatment of PNT-2 by BODIPY-ceramide, PKH67-labeled PC-3M-luc cells were added. After co-culturing for 3 or 12 h, images were obtained. Fluorescent photos were detected with the Eclipse TE 2000 Inverted Research Microscope, and images were produced using NIS-Elements BR software. *Arrowheads* show *yellow colored* cancer cells. The *size bar* indicates 100 μm.

of exosomes. We collected two separate aliquots of CM from PNT-2 cells incubated with or without GW4869, a specific inhibitor for neutral sphingomyelinase 2. The isolated exosomes were verified by the detection of CD63 protein, a well established exosome marker, with immunoblotting (supplemental Fig. 1*B*), and the activity of GW4869 was confirmed by the decreased amount of exosomal protein (supplemental Fig. 1*C*). The CM prepared in the presence of the GW4869 compound cancelled most tumor-suppressive activity of the non-treated PNT-2 CM (Fig. 1*B*; compare *lanes 1–3*). Furthermore, proliferation of PC-3M-luc cells was inhibited by the addition of the exosome fraction isolated from the PNT-2 CM by ultracentrifugation (Fig. 1*C*). These observations suggest that exosomal miRNAs derived from non-cancerous cells were transferred to cancerous cells, resulting in the inhibition of their proliferation.

To visualize the transfer of ceramide-containing exosome from PNT-2 to PC-3M-luc *in vitro*, a co-culture experiment was performed. Before the co-culture, 2×10^5 PNT-2 cells were incubated for 30 min with red fluorescent BODIPY-ceramide dye, which can label the exosomes inside the cells (13, 14). After washing five times with PBS, equal numbers of PC-3M-luc cells labeled by green fluorescent PKH67, a cellular membrane indicator, were added into the culture dishes. Three hours later we did not observe any PC-3M-luc cells with a yellow color (*Merged* photo in *upper panel* of Fig. 1*D*), indicating that car-

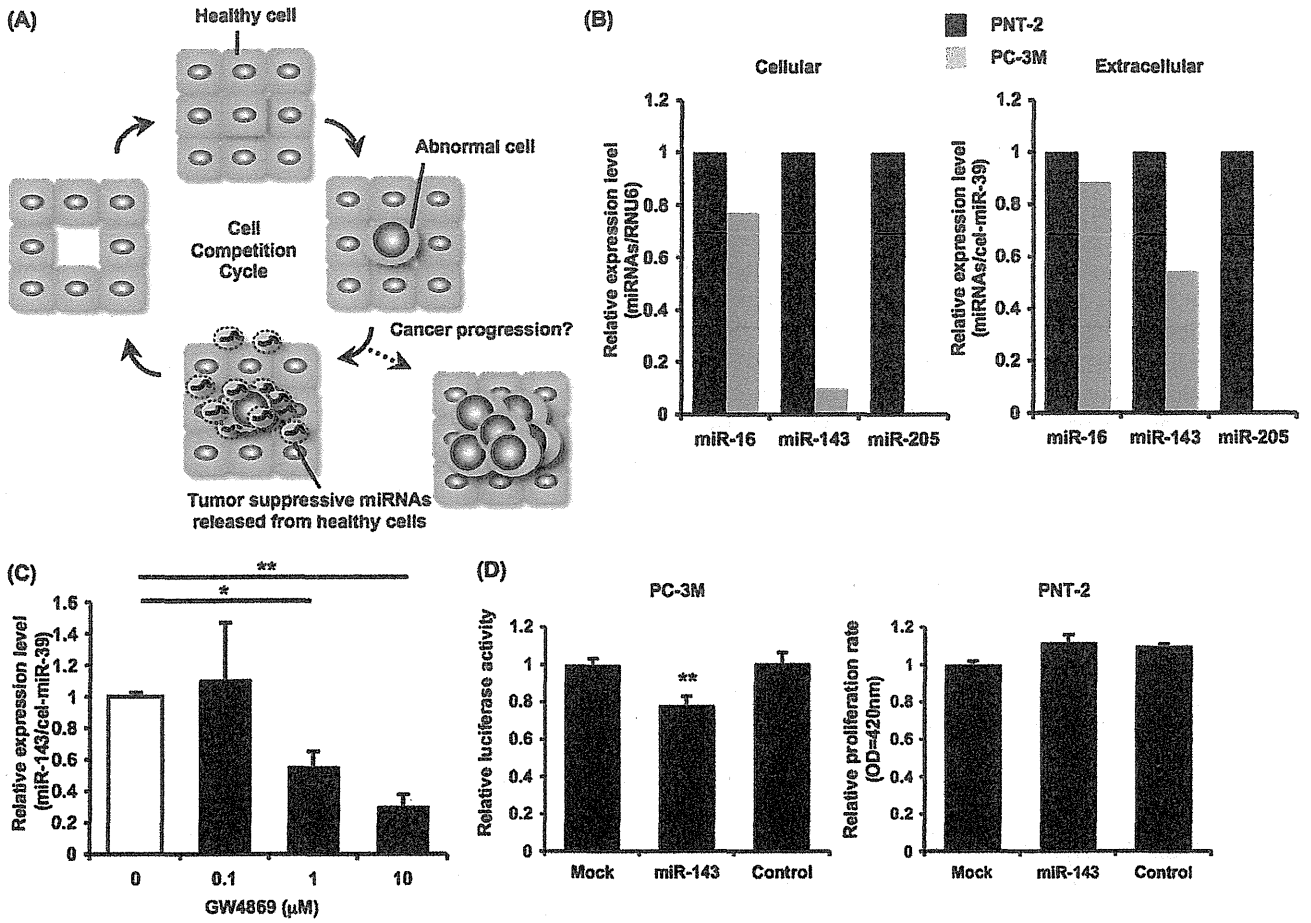

FIGURE 2. **Down-regulation of cellular and extracellular tumor-suppressive miRNAs in PC-3M-luc cells.** *A*, shown is a schematic representation of hypothetical tumor initiation process. Neighboring healthy cells (*blue*) secrete tumor-suppressive miRNAs (*light yellow*) to inhibit the proliferation of abnormal cells (gray), and this cell population returns to the initial healthy condition (a homeostatic cycle). Once the cell competitive cycle is compromised, this niche become susceptible to tumor initiation (indicated by a *dashed arrow*). *B*, comparison of cellular and extracellular miRNAs expression in PNT-2 and PC-3M-luc cells is shown. miRNA expression levels were determined by a Taq-Man QRT-PCR. The values on the *y* axis are depicted relative to the normalized expression level of PNT-2 cells, which is defined as 1. *C*, secretion of miR-143 was suppressed by the treatment with GW4869. PNT-2 cells were seeded and cultured in a 24-well plate for 48 h in the indicated concentrations of GW4869. After the incubation, the medium was subjected to QRT-PCR for miR-143. The values on the *y* axis are depicted relative to the amount of miR-143 at 0 μM GW4869, which is defined as 1. *D*, shown is cell growth inhibition by miR-143 in PC-3M-luc cells but not in PNT-2 cells. PNT-2 and PC-3M-luc cells were transfected with 10 nM miR-143 molecules (miR-143) or 10 nM negative control molecules (control) or without RNA molecules (*Mock*). The values on the *y* axis are depicted relative to the normalized luciferase activity of untreated cells (*Mock*), which is defined as 1. Each *bar* is presented as the mean S.E. ($n = 3$). *, $p < 0.05$; **, $p < 0.005$, as compared with untreated PC-3M-luc cells; Student's *t* test.

ried-over red dyes were thoroughly removed as 3 h is enough time for the dye to be incorporated directly into the cells. By contrast, after 12 h of co-culture, yellow fluorescence was observed in green-labeled PC-3M-luc cells (indicated by *arrowheads* in Merged photo in the *lower panel* of Fig. 1*D*), suggesting that ceramide-containing exosomes from PNT-2 cells were transferred to the PC-3M-luc cells. This result is corroborated by the uptake experiment using the PKH67-labeled exosomes purified from PNT-2 culture medium (supplemental Fig. 1*D*). Green fluorescence was detected in PC-3M-luc cells after 16 h of incubation, providing a direct evidence for exosome uptake by cancerous cells.

Tumor-suppressive miRNAs Down-regulated in Cancerous Cells Were Secreted from Non-cancerous Cells—We propose a hypothetical model of tumor initiation involving cell competition and anti-proliferative secretory miRNAs (Fig. 2*A*). In a cell competition cycle, as illustrated in the *bottom part* of Fig. 2*A*, growth inhibitory miRNAs are actively released from non-cancerous cells to kill abnormal cells with a partial oncogenic ability, thereby restoring them to a healthy state. Indeed, inhibitory capacity of these miRNAs appears to be limited in the setting of single treatment with the PNT-2 CM (Fig. 1*A*); however, they can potentially prevent emergence of tumor cells in a physiological condition. Because abundantly existing healthy cells continuously provide nascent overproliferative cells with tumor-suppressive miRNAs for a long period, a local concentration of secretory miRNAs can become high enough to restrain a tumor initiation. A *dashed arrow* in Fig. 2*A* indicates the way whereby the disruption of the homeostatic system leads to tumor expansion. If precancerous cells acquire resistance to anti-proliferative secretory miRNAs or normal cells cannot supply an adequate amount of miRNAs, then this defensive system will fail to maintain the healthy condition.

To test this hypothesis we checked the secretion amount of representative tumor-suppressive miRNAs by comparing PNT-2 and PC-3M-luc cells with Taq-Man QRT-PCR analysis. As shown in Fig. 2*B*, miR-16, miR-205, and miR-143, which are already reported to be dysregulated in prostate cancer (10, 15, 16), were down-regulated in PC-3M-luc cells at a cellular and extracellular level. The GW4869 inhibitor suppressed the secretion of miR-143 from PNT-2 cells in a dose-dependent manner (Fig. 2*C*), whereas its cellular level was not altered (supplemental Fig. 2*A*). Additionally, the application of small interfering RNAs specific for human neutral sphingomyelinase 2 gene knocked down its mRNAs, resulting in profound decrease in miR-143 secretion (supplemental Fig. 2, *B* and *C*). On the contrary, the expression of miR-143 in the cells was not changed after the transfection of neutral sphingomyelinase 2 siRNA (supplemental Fig. 2*D*). Taken with the result of Fig. 1*B*, these results suggest that the secreted tumor-suppressive miRNAs are implicated in the process of growth inhibition by PNT-2 CM.

For a global understanding of the expression change of non-cancerous and cancerous cells, we performed an miRNA microarray analysis against cellular and exosomal RNAs purified from PNT-2 and PC-3M-luc cells. In the sub-dataset of secretory exosomal miRNAs from PNT-2 cells, we found 40 miRNAs whose cellular amounts were lowered by one-half in PC-3M-luc cells (Table 1). The selected miRNAs expectedly include several types of tumor-suppressive miRNAs, such as miR-15a, miR-200 family, miR-148a, miR-193b, miR-126, and miR-205 (10, 15, 17–20). This observation supports the idea that secretory tumor-suppressive miRNAs are transferred from non-cancerous cells to cancerous cells, in accordance with the concentration gradient of the miRNA.

We have so far demonstrated that normal cells have a higher secretion of tumor-suppressive miRNAs than cancerous cells; however, it remains unclear whether or not these secreted miRNAs affect the proliferation of cells of their origin. To answer this question, we introduced synthesized miR-143 to both PNT-2 and PC-3M-luc cells and assessed their proliferation rates. After 3 days of transfections, the miR-143 analog induced growth inhibition of PC-3M-luc cells compared with mock and control small RNA transfection (Fig. 2*D*, *left panel*). In contrast, the exogenously transduced miR-143 did not show its anti-proliferative effect in PNT-2 cells (Fig. 2*D*, *right panel*), indicating that excessive miR-143 did not confer an additional growth inhibitory effect on normal cells in which expression of miR-143 is maintained to a physiological level. This finding suggests that animal cells may have their own threshold amount for miRNA activity. The different sensitivity found in different cell types can help secretory miRNAs fulfill their purpose to combat exclusively precancerous cells. It is possible that secretory miRNAs, at least, derived from non-cancerous cells such as PNT-2 cells could supplement growth-suppressive signals that are decreased in cancerous cells. Thus, secreted miR-143 might be involved in the cell competitive regulatory system.

TABLE 1
A list of PNT-2-derived secretory miRNAs that were down-regulated less than 0.5-fold in PC-3M cells compared with PNT-2 cells

miRNAs	Fold change[a]
hsa-miR-141	0.0
hsa-miR-200c	0.0
hsa-miR-886–3p	0.0
hsa-miR-30a*	0.0
hsa-miR-155	0.0
hsa-miR-205	0.0
hsa-miR-224	0.0
hsa-miR-148a	0.0
hsa-miR-130a	0.0
hsa-miR-30a	0.1
hsa-miR-663	0.1
hsa-miR-181a-2*	0.1
hsa-miR-484	0.1
hsa-miR-10a	0.1
hsa-miR-192	0.1
hsa-miR-193b	0.1
hsa-miR-200a	0.1
hsa-miR-429	0.1
hsa-miR-769–5p	0.1
hsa-miR-200b	0.2
hsa-miR-195	0.2
hsa-miR-203	0.2
hsa-miR-7	0.2
hsa-miR-200a*	0.2
hsa-miR-200b*	0.2
hsa-miR-30c	0.2
hsa-miR-126	0.3
hsa-miR-149	0.3
hsa-miR-30d	0.3
hsa-miR-181a	0.3
hsa-miR-30e*	0.3
hsa-miR-365	0.4
hsa-miR-135b	0.4
hsa-miR-454*	0.4
hsa-miR-129*	0.4
hsa-miR-30b	0.4
hsa-miR-181b	0.4
hsa-miR-210	0.4
hsa-miR-455–3p	0.5
hsa-miR-15a	0.5

[a] Fold change of the expression of miRNAs in PC-3M cells compared with PNT-2 cells is indicated.

Secretory miR-143 Inhibited Prostate Cancer Cell Proliferation in Vitro—To examine whether miR-143 released from normal cells exert an anti-proliferative activity, we generated HEK293 cells overexpressing miR-143 by nearly 200-fold compared with control (supplemental Fig. 3*A*). After a 3-day incubation with the CM derived from the miR-143-overproducing HEK293 cells and control HEK293 cells, PC-3M-luc cells showed an ~50% decrease in proliferation (Fig. 3*A*, *lanes 1* and *3*). Importantly, the decrease was recovered by the transfection of anti-miR-143 in PC-3M-luc cells (Fig. 3*A*, *lane 3* and *4*). These data indicate that the growth inhibition is attributable to secretory miR-143 contained in the supernatant of miR-143-overexpressing HEK293 cells. In agreement with the exosome-dependent machinery of miRNA secretion, we observed a similar result by using exosome fractions purified from miR-143-transduced HEK293 cells (Fig. 3*B*).

To further study miRNA transfer on a molecular level, we performed a target gene expression analysis and an miRNA-responsive reporter assay. The immunoblotting analysis shows that the addition of the CM isolated from miR-143-overexpressing HEK293 cells significantly knocked down expression of KRAS, a target gene for miR-143 (21), in PC-3M-luc cells (Fig. 3*C*). In addition, we implemented luciferase analyses using

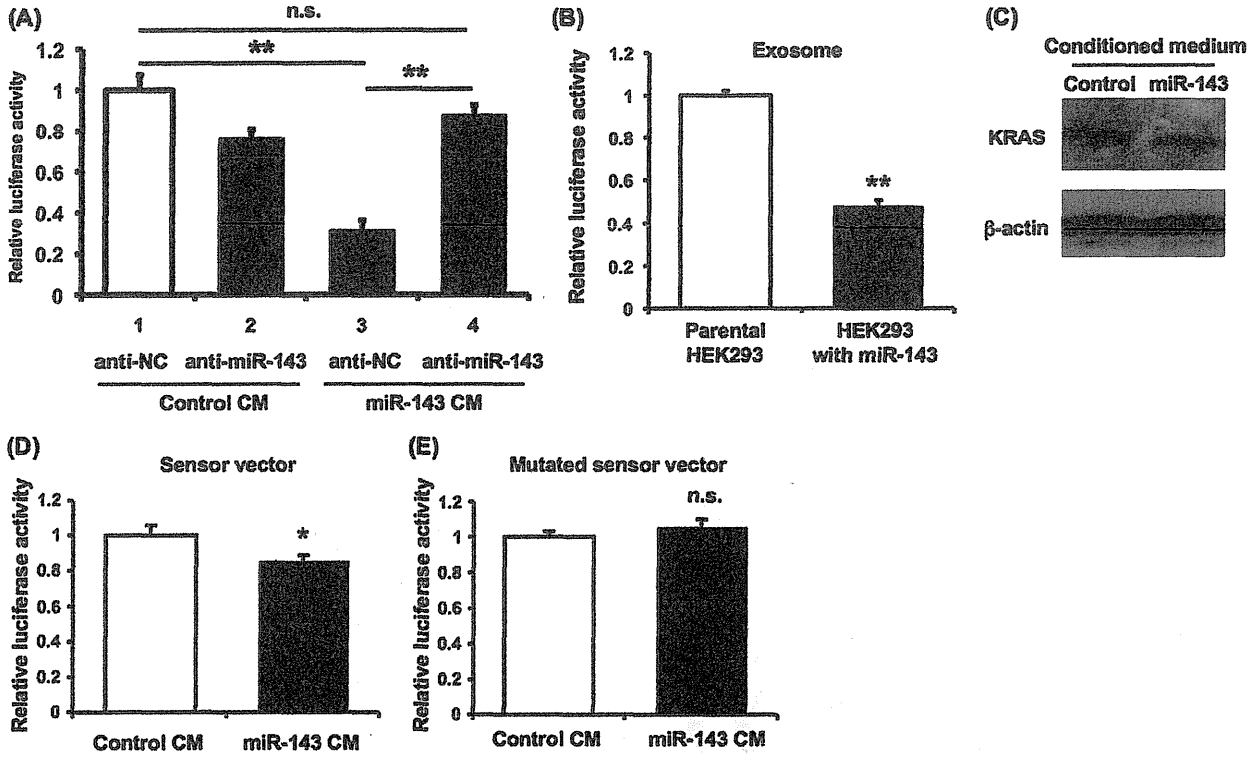

FIGURE 3. **Transfer of secretory miR-143 to PC-3M-luc cells *in vitro*.** *A*, the transfection of anti-miR-143 to PC-3M-luc cells restored the reduced cell growth by the CM derived from miR-143 overproducing cells. After the transfection with 3 nM miR-143 inhibitor molecule (anti-miR-143) (*lanes 2* and *4*) or its control molecule (*anti-NC*) (*lanes 1* and *3*), PC-3M-luc cells were incubated for 3 days in a control conditioned medium (*lanes 1* and *2*) and CM containing extracellular miR-143 (*lane 3* and *4*) followed by a cell growth assay as described under "Experimental Procedures." The values on the *y* axis are depicted relative to the normalized luciferase activity of cells treated in a culture medium, which is defined as 1. Each *bar* is presented as the mean S.E. ($n = 3$). (*, $p < 0.05$; Student's *t* test; *n.s.*, not significant). *B*, cell growth inhibition by exosomes derived from miR-143-transduced HEK293 cells is shown. PC-3M-luc cells were incubated in the exosomes followed by cell growth assay as described under "Experimental Procedures." The values on the *y* axis are depicted relative to the normalized luciferase activity of cells treated with exosomes derived from original HEK293 cells, defined as 1. Each *bar* is presented as the mean S.E. ($n = 3$). (**, $p < 0.005$; Student's *t* test). *C*, secretory miR-143-mediated KRAS suppression in PC-3M-luc cells is shown. Ten micrograms of protein of whole cell lysates prepared from PC-3M-luc cells treated with or without secretory miR-143 were applied to electrophoresis. Immunoblotting was performed with KRAS and actin antibodies and visualized by LAS-3000 system. *D*, extracellular miR-143 derived from HEK293 cells suppressed the luciferase activity of the sensor vector. HEK293 cells transfected with an miR-143 sensor vector were used as recipient cells. The recipient cells were incubated in a CM containing extracellular miRNAs. After a 2-day incubation, a luciferase reporter assay was performed as described under "Experimental Procedures." The values on the *y* axis are depicted relative to the normalized luciferase activity of original HEK293-conditioned medium-treated cells, which is defined as 1. Each *bar* is presented as the mean S.E. ($n = 3$). *, $p < 0.05$; Student's *t* test). *E*, extracellular miR-143 did not reduce the luciferase activity of the mutated sensor vector. HEK293 cells transfected with the mutated miR-143 sensor vector were used as recipient cells. The recipient cells were incubated in a conditioned medium containing extracellular miRNAs. 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 *bar* is presented as the mean S.E. ($n = 3$). *n.s.* represents not significant.

a sensor vector harboring renilla luciferase fused in tandem with miR-143 seed sequence in the 3′-UTR. As shown in Fig. 3*D*, the normalized renilla luciferase activities were reduced by the treatment of miR-143-enriched CM derived from HEK293 cells stably expressing miR-143. In contrast, we did not detect any changes of luminescence by using a mutated vector instead of the intact sensor vector (Fig. 3*E*). Furthermore, we quantified cellular amounts of miR-143 in PC-3M-luc cells incubated with CM derived from HEK293 cells or miR-143 overproducing HEK293 cells by QRT-PCR. As shown in supplemental Fig. 3*B*, miR-143 was clearly increased at a cellular level by the treatment of the miR-143 enriched CM. These results indicate that secretory miR-143 exhibits its on-target growth-inhibitory effect in neighboring precancerous cells, thereby suppressing their disordered growth.

Secretory miR-143 Functions as Tumor Suppressor in Vivo—To our knowledge it has never been demonstrated that extracellular tumor-suppressive miRNAs can be transferred into living cells and induce phenotypic change *in vivo*. To address this possibility, we injected CM derived from miR-143 overproducing HEK293 cells or parental HEK293 cells into nude mice implanted with PC-3M-luc cells. Four days after the subcutaneous implantation, we carried out *in vivo* imaging and CM injections according to the timetable shown in Fig. 4*A*. Tumor expansions have been restrained for 8 days with intratumor administrations of miR-143 enriched CM, and consequently the tumor masses shrank by ~0.5-fold on day 8 (Fig. 4*B*). The representative luminescent images of inoculated PC-3M-luc cells on day 8 were shown in Fig. 4*C*. Consistent with the finding that miR-143 did not impair growth activity of non-cancer cells *in vitro* (Fig. 2*D*), no toxicity was observed in these mice (data not shown). In addition, the expressions of miR-143 target genes, such as KRAS and ERK5 (16, 21), were decreased after miR-143-transduced CM injections, indicative of intercellular miRNA transfer *in vivo* (Fig. 4*D*). Thus, our prostate cancer xenograft model suggests that the tumor-suppressive miRNAs

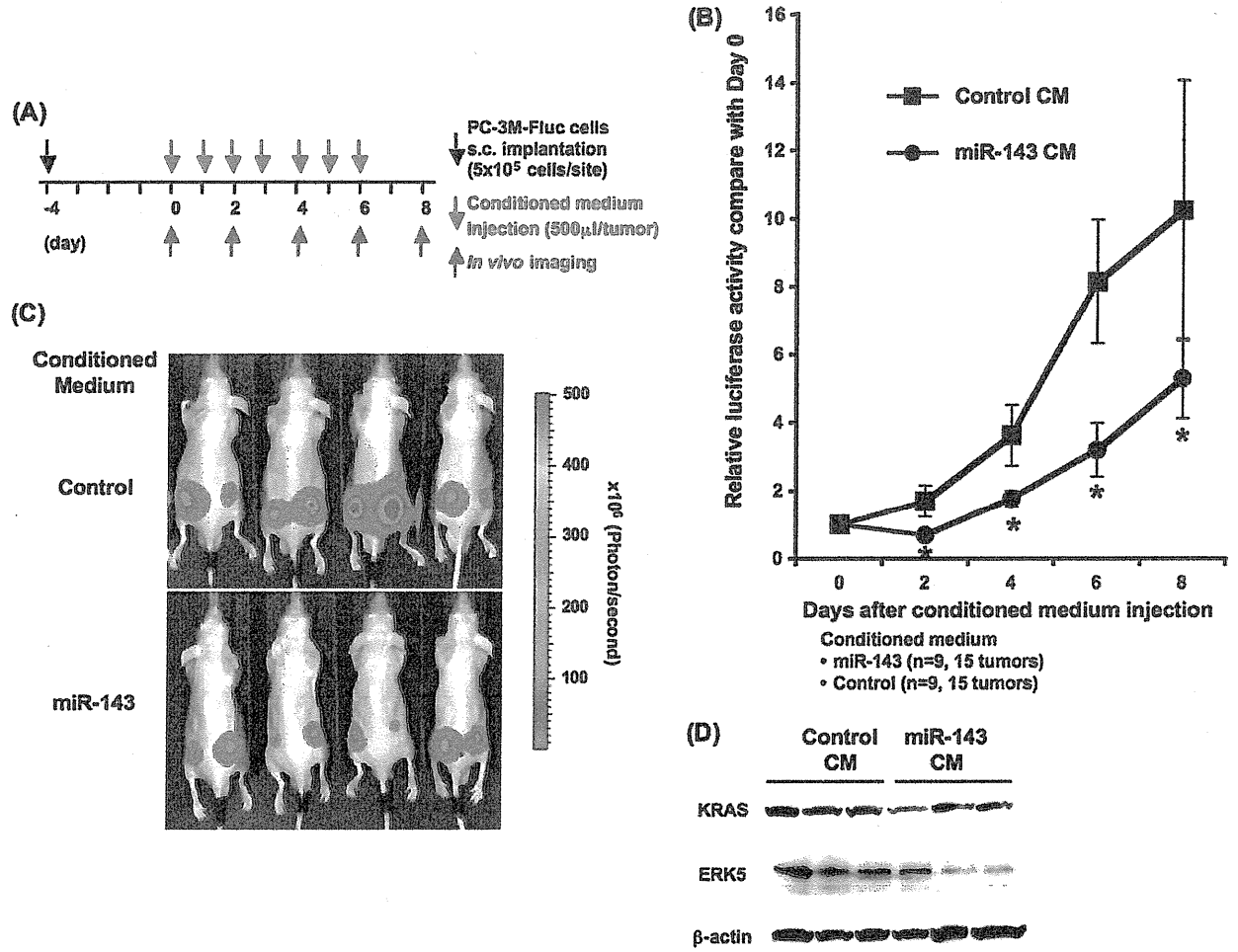

FIGURE 4. **Transfer of secretory miR-143 to PC-3M-luc cells *in vivo*.** *A*, shown is the timetable for conditioned medium injections and *in vivo* imaging. *B*, shown are tumor growth ratios of the inoculated PC-3M-luc cells during the secretory miR-143 treatment. *Closed circles* and *closed squares* indicate the tumor mass administrated with CM from miR-143-overproducing HEK293 cells or parental HEK293 cells, respectively. The values on the *y* axis are depicted relative to the luciferase activity of each tumor on day 0, which is defined as 1. Each *bar* is presented as the mean S.E. ($n = 9$). *, $p < 0.05$; Student's *t* test. *C*, representative images are shown of tumor cells in the skin of mice. Bioluminescence of firefly luciferase from miR-143-enriched CM treated mice and control mice were detected on day 8 with IVIS imaging system. *D*, shown is secretory miR-143-mediated KRAS and ERK5 suppression in inoculated tumor cells. On day 8 the inoculated tumor masses were isolated and applied to immunoblotting analysis for the quantification of KRAS and ERK5 on a protein level.

secreted from normal cells could be efficiently delivered into their neighboring tumors *in vivo*.

DISCUSSION

In this study we documented that miR-143 derived from non-cancerous cells had the ability to suppress the growth of cancer cell proliferation not only *in vitro* but also *in vivo*. These observations suggest that tumor-suppressive miRNAs can be implicated in cell competition between cancer cells and non-cancer cells. In this context, normal cells attempt to prevent the outgrowth of precancerous cells by secreting anti-proliferative miRNAs and maintain a healthy condition; however, the abnormal cells can circumvent this inhibitory machinery, finally resulting in a tumor expansion (Fig. 2*A*). Cell competition could be a homeostatic mechanism that tumor cells need to overcome (1).

Here, we discuss two possible mechanisms by which cancer cells can gain resistance to secretory tumor-suppressive miRNAs. One is a blockade for the uptake of miRNAs, and the other is a cancellation of silencing activity of the incorporated miRNAs. As previously reported, miRNAs are loaded into exosomes and then secreted from living cells (7, 22, 23). If exosomes enriched in miRNAs are actively incorporated by recipient cells, cancer cells can impair the uptake mechanism to escape from the attack of secretory tumor-suppressive miRNAs. This scenario is supported by a recent publication regarding a Tim4 expected for an exosome receptor (24).

In the latter case cancer cells need to specifically compromise the incorporated tumor-suppressive miRNAs because there are some types of miRNAs that are indispensable for the expansion of cancer cells. A RISC assembly is composed of many protein families, such as the mammalian AGO family, GW182, and heat shock proteins (25). Moreover, each gene family also consists of many members, thereby generating diversity of RISC assemblies. The heterogeneity of RISC assemblies allows tumor-suppressive miRNAs to selectively bind with a RISC and silence their target genes on the complex. If cancer cells can exclusively destroy the tumor-suppressive RISC assembly, they can safely

grow in a limited niche full of anti-proliferative miRNAs. The detailed mechanism of the resistance to cell competition remains unknown.

In addition to the acquired resistance, there is another possibility that normal cells will lose secretory capacity of exosomal miRNAs. p53 was shown to enhance exosome production in cells undergoing a p53 response to stress (26). In other words, dysfunction of p53 will result in decreased miRNA secretion. The tumor-suppressive ability of p53 can partly depend on the control of miRNA release from normal cells.

Numerous studies show a broad variety of reasons for tumor initiation, including gene amplification, cellular stress, metabolic alteration, and epigenetic changes. This work suggests that the disruption of the cell competitive process mediated by secretory miRNAs will result in the occurrence of neoplasm. Understanding the mechanism by which homeostasis is impaired leads to a novel therapeutic approach for cancer progression.

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Unraveling the mystery of cancer by secretory microRNA: horizontal microRNA transfer between living cells

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microRNAs (miRNAs) have been identified as a fine-tuner in a wide array of biological processes, including development, organogenesis, metabolism, and homeostasis. Deregulation of miRNAs causes diseases, especially cancer. This occurs through a variety of mechanisms, such as genetic alterations, epigenetic regulation, or altered expression of transcription factors, which target miRNAs. Recently, it was discovered that extracellular miRNAs circulate in the blood of both healthy and diseased patients. Since RNase is abundant in the bloodstream, most of the secretory miRNAs are contained in apoptotic bodies, microvesicles, and exosomes or bound to the RNA-binding proteins. However, the secretory mechanism and biological function, as well as the significance of extracellular miRNAs, remain largely unclear. In this article, we summarize the latest and most significant discoveries in recent peer-reviewed research on secretory miRNA involvement in many aspects of physiological and pathological conditions, with a special focus on cancer. In addition, we discuss a new aspect of cancer research that is revealed by the emergence of "secretory miRNA."

Keywords: secretory microRNA, microRNA, exosome, cell–cell communication, cancer

INTRODUCTION

microRNAs (miRNAs) are small non-coding RNA that repress a wide variety of target genes expression at the post-transcriptional level by sequence-specific base pairing to the 3′ untranslated region of multiple target mRNAs. They are conserved through species, and form an important class of regulators that participate in multiple biological phenomena, including development, organogenesis, and homeostasis. Because of their ability to bind to many target mRNAs (Kwak et al., 2011), once their expression is altered, disease could occur through the deregulation of their target gene networks, particularly that leading to cancer. For this reason, many recent studies have focused on the development of novel diagnosis and therapeutics in the field of oncology. Current studies have revealed that miRNAs are secreted outside of the cells, and their biological significance is beginning to be recognized (Zernecke et al., 2009; Kosaka et al., 2010b; Pegtel et al., 2010). This article is a summary of the latest and most significant findings of original studies on the involvement of secretory miRNAs in cancer, with a special focus on the potential of secretory miRNAs as a humoral factor for cancer biology.

RNA IS NOT ONLY THE MEDIATOR IN THE CENTRAL DOGMA BUT ALSO A SECRETORY FUNCTIONAL MOLECULE

Before Watson and Crick (1953) described the double-helical structure of the DNA molecule, Mandel and Metais (1947) had found that DNA is present in plasma and serum in 1947. They showed the presence of nucleic acids in healthy subjects as well as in ill patients. After that, many researchers have tried to examine the circulating nucleic acid to develop them as a potential biomarker, especially in the research field of cancer (Fleischhacker and Schmidt, 2007). It is now well documented that RNA can also be detected in plasma, serum, and other body fluids as well as from cell-free supernatants of *in vitro* cultivated cells. One of the first papers demonstrating the presence of extracellular RNA was published by Stroun et al. (1978). They reported the presence of an RNA form in a nucleoprotein complex spontaneously released from human blood lymphocytes and frog cell systems from auricle cultures. They also showed that the RNA from this complex has a stimulating effect on DNA synthesis *in vitro*, suggesting the function of secretory RNA in recipient cells.

Meanwhile, the uptake of RNA by recipient cells was also observed. More than 40 years ago, RNAs were reported to be readily taken up by ascites tumor cells (Galand and Ledoux, 1966). In addition, during a study of co-cultured cells that were previously incubated with or without tantalum particles, intact labeled RNA was found to be transferred into the non-labeled recipient cells from labeled donor cells (Kolodny, 1971). Namely, cell–cell communication was mediated not only by proteins, such as cytokines, chemokines, and hormones, but also by secretory RNA.

Given that the concentration of RNA-degrading enzymes, RNase, is high in normal people and even higher in cancer patients (Reddi and Holland, 1976; Tsui et al., 2002) and that RNase is extremely stable, it was reasoned that the RNA released from the cells into the extracellular space must be complexed and in a form that is resistant against RNases. The first study of associating circulating RNA, as RNA–proteolipid complexes, in serum was reported in 1987 (Wieczorek et al., 1987). This study reported a relationship between the presence of RNA–proteolipid complexes and tumor mass/response to therapy. These complexes disappeared ~48 h after tumor removal and were undetected in benign disorders. Another study demonstrated that the release of a macromolecular substance containing ^{32}P and ^{3}H was found when prelabeled

Chinese hamster ovary cells were treated with trypsin under conditions in which cells remain fully viable (Rieber and Bacalao, 1974). In contrast, a ribonuclease treatment affected neither the ^{32}P nor the ^{3}H radioactivity. The authors concluded from these experiments that RNA together with glycoproteins is released from the external cell surface.

FUNCTIONAL IMPORTANCE OF SECRETORY miRNA IN VARIOUS KINDS OF LIFE PHENOMENA

miRNAs, a class of post-transcriptional gene expression regulators, play critical roles in various kinds of biological phenomena, including development, organogenesis, and homeostasis. Dysregulation of miRNA leads to cancer development and progression and has different expression profiles in normal tissues and cancers (Garzon et al., 2010). For this reason, miRNAs have been investigated for their potential use in the diagnosis, prognosis, and treatment of cancer. miRNAs have recently been detected in human body fluids, including peripheral blood plasma as extracellular nuclease-resistant entities (Kosaka et al., 2010a). Reports in two landmark papers noted that not only mRNAs but also miRNAs were secreted outside of the cells and circulated in human body fluid (Chim et al., 2008; Lawrie et al., 2008). Chim et al. (2008) reported the existence of placental miRNAs in maternal plasma. Interestingly, they showed that the four most abundant placental miRNAs (miR-141, miR-149, miR-299-5p, and miR-135b) were detectable in maternal plasma during pregnancy and showed reduced detection rates in post-delivery plasma. Furthermore, Lawrie et al. (2008) investigated whether miRNAs have diagnostic utility by comparing the levels of tumor-associated miR-155, miR-210, and miR-21 in serum from diffuse large B-cell lymphoma patients with healthy controls and showed that the levels were higher in patients than in control sera. These observations support the idea that circulating miRNAs can be used as biomarkers to monitor an individual's health. In addition, these reports also suggest the possibility that secretory miRNA must be contained in or attached to something that could protect RNA from RNase-mediated degradation.

One breakthrough about circulating RNA was the discovery of mRNA and miRNA in exosomes (Valadi et al., 2007). Valadi et al. (2007) showed that mouse and human mast cell-derived exosomes, which are vesicles of endocytic origin released by many kinds of cells that can mediate communication between cells, contain RNA and miRNA. The RNA from mast cell exosomes is transferable to other mouse and human mast cells. After the transfer of mouse exosomal RNA to human mast cells, new mouse proteins were found in the recipient human cells, indicating that transferred exosomal mRNA can be translated after entering another cell. Observations from these three reports indicated one important fact, namely, that miRNA could be existent in the outer space of the cells, where the RNase is present, and could be functional in this new location.

After the discovery of miRNA in exosome, many researchers attempted to identify the function of secretory miRNA because the report from Valadi et al. (2007) had not clarified it in the exosomal miRNA in recipient cells. One of the earliest studies to prove the function of secretory miRNA was revealed by an apoptotic body (Zernecke et al., 2009). They demonstrated that CXCL12 production was mediated by miR-126, which was enriched in apoptotic bodies and repressed the function of the regulator of G protein signaling 16. This enabled CXCR4 to trigger an autoregulatory feedback loop that increased the production of CXCL12, leading to the recruitment of progenitor cells. This study strongly indicated the importance of a "dying message" for the regulating homeostasis of a healthy status and highlights the functions of miRNAs in health and disease that may extend to the recruitment of progenitor cells during other forms of tissue repair or homeostasis.

After the study of miRNA in apoptotic bodies, three reports showed the function and transfer of secretory miRNAs contained inside the exosome. Pegtel et al. (2010) showed that mature EBV-encoded miRNAs are secreted by EBV-infected B cells through exosomes. These EBV-miRNAs repress the EBV target immunoregulatory genes, and these target genes are down-regulated in primary EBV-associated lymphomas. Interestingly, using peripheral blood mononuclear cells from patients with an increased EBV load, these researchers also showed that, although EBV DNA is restricted to the circulating B-cell population, EBV BART miRNAs are present in both B-cell and non-B-cell fractions, suggestive of miRNA transfer *in vivo*. Zhang et al. (2010) reported that miR-150 is contained inside the exosomes and is secreted from a cultured human monocyte/macrophage cell line and that this exosome delivers miR-150 into human microvascular endothelial cells. Then, elevated exogenous miR-150 effectively reduced c-Myb expression and enhanced cell migration in human microvascular endothelial cells. Our group also demonstrated that a secreted tumor-suppressive miRNA, which is miR-146a down-regulated in prostate cancer, was transported to cancer cells and exerted gene silencing in the recipient prostate cancer cells through the suppression of its target gene, thereby leading to cell growth inhibition (Kosaka et al., 2010b). This suggested that secreted miRNA could function as a cell–cell communication tool between the cancer cells and their microenvironmental cells.

These three reports clarified a variety of physiological and pathological phenomena, including virus infection, vascular disease, and cancer. The variety of research fields highlights the importance of secretory miRNAs in phenomena vital to life. Indeed, recent reports have pointed to various functions of secretory miRNA in many aspects of life, such as cellular communication involving antigen-dependent, unidirectional intercellular transfer of miRNAs by exosomes during immune synapsis (Mittelbrunn et al., 2011), nasopharyngeal carcinoma-mediated transfer of EBV-encoded BART miRNA (Gourzones et al., 2010), hepatocellular carcinoma (Kogure et al., 2011), and cardiovascular diseases (Kuwabara et al., 2011). These reports mainly described the importance of exosomes as an miRNA carrier; however, it is not always the exosome that is important in secretory miRNA-mediated cell–cell communication.

High-density lipoprotein (HDL) transports endogenous miRNAs and delivers them to recipient cells with functional targeting capabilities (Vickers et al., 2011). The human HDL-miRNA profile of normal subjects is significantly different from that of familial hypercholesterolemia subjects. Interestingly, a recent report showed that the mechanism of horizontal transfer of miRNAs is not only dependent on vesicle transfer, such as exosomes, but

also intercellular connection tools, such as gap junction and RNA-binding protein. Lim et al. (2011) clarified that miRNA was transmitted from bone marrow stroma to breast cancer cells via gap junctions and exosomes in tumor cell quiescence. Arroyo et al. (2011) employed a technique, differential centrifugation and size-exclusion chromatography, to characterize circulating miRNA complexes in human plasma and serum and found that the majority of circulating miRNAs cofractionated with Argonaute2 (Ago2, the key effector protein of miRNA-mediated silencing) protein complexes rather than within vesicles. This study was also confirmed by other groups which have shown Ago2 (Turchinovich et al., 2011) or nucleophosmin 1 as secretory miRNA carriers (Wang et al., 2010). Further biological studies are required to understand the function of miRNAs secreted with an RNA-binding protein, such as Ago2 or nucleophosmin 1, in a variety of research fields.

To certify the significance of secretory miRNAs in variety of life phenomena, it is also essential to understand the secretion mechanism of miRNA from cells. Previously, we found in HEK293 and COS-7 cells that the secretion of miRNAs was regulated by neutral sphingomyelinase 2 (nSMase 2; Kosaka et al., 2010b), which is the catalytic enzyme of ceramide biosynthesis and is known as an exosome regulatory protein (Trajkovic et al., 2008). The decreased activity of nSMase 2 with a chemical inhibitor, GW4869, and a specific siRNA resulted in the reduced secretion of miRNAs. Complementarily, overexpression of nSMase 2 increased the extracellular amounts of miRNAs. This observation was also confirmed using other cells including T-cells (Mittelbrunn et al., 2011) and hepatocellular carcinoma cells (Kogure et al., 2011). Contrary to our results, inhibition of nSMase 2 significantly increased the amount of miRNAs exported to HDL from macrophages (Vickers et al., 2011).

It remains necessary to elucidate how miRNA is sorted into exosomes or other vesicles, such as microvesicles. Microvesicles, also known as microparticles or shedding vesicles, represent a heterogeneous population of vesicles with a diameter of 100–1000 nm that are released by budding of the plasma (Muralidharan-Chari et al., 2010). It has been shown that microvesicles isolated from embryonic stem cells increase pluripotency of hematopoietic stem cells after horizontal transfer of embryonic stem cell-derived mRNA. Although the functions of microvesicles were recently elucidated, unlike exosome, not only the function but also the sorting mechanisms of miRNAs into microvesicles have not been clarified yet. Furthermore, it has not been shown yet what kind of protein bind to miRNAs in the vesicles such as exosomes, microvesicles, and apoptotic bodies, although Arroyo et al. (2011) clearly showed that circulating Ago2-binding miRNAs were not contained inside vesicles. Gibbings et al. (2009) detected some AGO2 in the purified exosomes, albeit less than in whole-cell lysates, on the contrary, GW182, which required for miRNA function through its binding to AGO2, was dramatically enriched in exosomes. Detecting the proteins, which bind to miRNAs in vesicles, might lead to revealing the sorting mechanism of miRNAs in vesicles. Clarifying the details of the molecular mechanisms of secretory miRNA, such as the manner of cell–cell transfer or secretion mechanisms, will help us understand a variety of diseases, especially cancer (**Figure 1**).

SECRETORY miRNA AS A HUMORAL FACTOR IN CANCER CELLS

As shown in this report, secretory miRNAs are functional molecules that modulate many aspects of the biological process. In addition, destruction of the secretion of miRNA from cells might lead to disease, such as cardiovascular diseases, virus infections, deterioration of the immune system, and cancer. From the field of cancer research, we would like to propose two hypotheses regarding secretory miRNA-mediated cancer progression (**Figure 2**).

One is the function of secretory miRNA in a metastatic niche (**Figure 2A**). As already shown in several reports, various types of the cells have been shown to have the capability to take up exosomes. The tumor microenvironment is a complex tissue comprising variable numbers of tumor cells, epithelial cells which originated cancer cells, fibroblasts, endothelial cells, and infiltrating leukocytes. Recent reports have explained the mechanism of controlling the cancer cell-mediated phenotypical change of microenvironmental cells through cytokines (Hanahan and Weinberg, 2011). Cytokines are considered as key molecules controlling autocrine or paracrine communications within and between these individual cell types. However, considering the existence of secretory miRNA within these environments, their influence to the cancer niche should be reconsidered. An exosome contains nearly 300 proteins (Atay et al., 2011) with the potential to modulate the state of microenvironment cells. In addition, miRNAs are known to regulate hundreds of target mRNA expressions. Thus, not only exosomal miRNAs but also other types of secretory miRNAs could control the state of cellular phenotypes to the benefit of cancer cells within their niche.

Another hypothesis deals with the function of secretory miRNAs in distant organs (**Figure 2B**). Recently, Hood et al. (2011) provided evidence of exosome-mediated conditioning of lymph nodes and defined microanatomic responses that enable the metastasis of melanoma cells. Homing of melanoma exosomes to sentinel lymph nodes imposes synchronized molecular signals that affect melanoma cell recruitment, extracellular matrix deposition, and vascular proliferation in the lymph nodes. They showed the physiological importance of exosomes for distal metastasis; however, they have not provided evidence of the molecules species that take part in the modulation of the distal site of metastasis. To reveal the exact function of miRNA targeting sites that are distant from the primary organ, we should identify the molecular mechanisms of the tropism of secretory miRNA transported by carriers.

SECRETORY miRNA AS A HUMORAL FACTOR IN ORGANISMS

In this study, systemic transfer of miRNAs has been introduced. However, an active mechanism for the transport of double strand RNA (dsRNA) across tissues and cellular boundaries was found in other organisms, such as nematode and plant. Transmembrane channel-forming protein SID-1 has been shown to mediate passive cellular uptake and cell-to-cell distribution of dsRNA in the nematode *C. elegans* (Feinberg and Hunter, 2003). In addition, recent report showed that mammalian SID-1 homolog localized to the cell membrane of human cells enhances their uptake of small interfering RNA, resulting in increased siRNA-mediated gene silencing efficacy (Duxbury et al., 2005). Furthermore, although RNA molecules have been implicated in systemic cell-to-cell communication

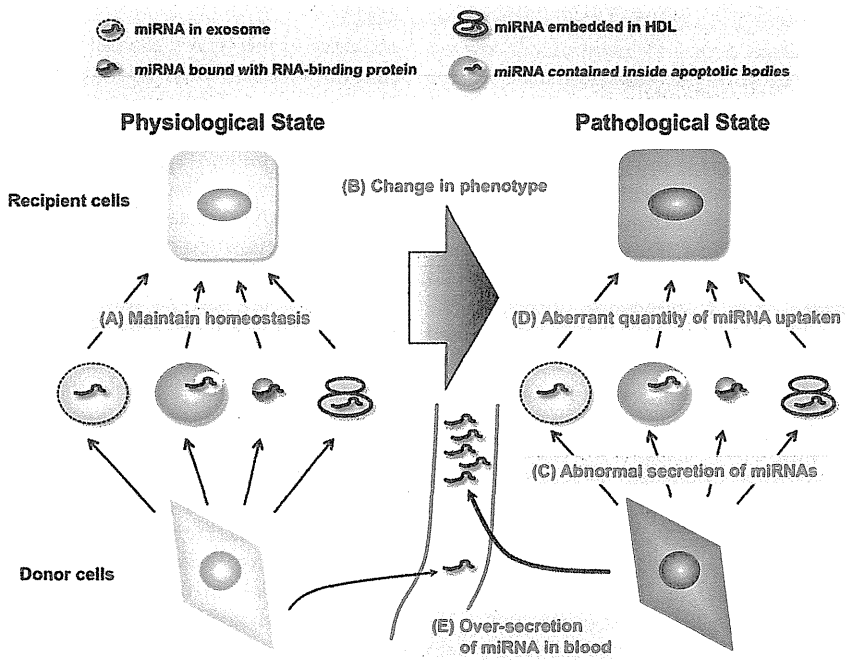

FIGURE 1 | Unveiling the mechanisms and roles of secretory miRNAs in both physiological and pathological conditions is essential for the development of novel diagnostic methods and therapeutics to treat various human diseases. In the physiological condition, secretory miRNAs may maintain the homeostasis of organs and tissues *via* diverse types of miRNA carriers **(A)**. However, this state may be destroyed by the change of phenotype in donor cells or recipient cells **(B)**. From the viewpoint of donor cells, changes in the phenotype of donor cells may lead to the alteration of miRNA species with carriers **(C)**. Furthermore, the quantity of secretory miRNA uptake by recipient cells may change in this situation **(D)**. It is necessary to understand these abnormal changes of molecular mechanisms for the development of a therapy against "secretory miRNAs-mediated diseases." In addition, it is possible that the amount of secretory miRNAs changes in this situation through the abnormal regulation of carrier production **(E)**. Understanding these changes may lead to the exploitation of a diagnosis marker for "secretory miRNAs-mediated diseases."

in plants (Chitwood and Timmermans, 2010), recent studies have shown that miRNAs are mobile signals that control gene expression during plant development (Dunoyer et al., 2010; Molnar et al., 2010), suggesting that the transfer of RNA is found globally in organisms.

Surprisingly, Zhang et al. (2011) reported that exogenous plant miRNAs are present in the sera and tissues of various animals and that these exogenous plant miRNAs are primarily acquired orally, through food intake. Rice abundant miRNA, miR-168a, is one of the most highly enriched exogenous plant miRNAs in the sera of Chinese subjects. Furthermore, they also found that MIR168a could bind to the human/mouse low-density lipoprotein receptor adapter protein 1 (LDLRAP1) mRNA, inhibit LDLRAP1 expression in liver, and consequently decrease LDL removal from mouse plasma. This study prompted the idea that miRNAs could regulate the gene expression across the kingdom. In addition, one of the important point of this study is that identification of plant miRNAs in human peripheral blood was performed by Solexa sequencing. High-throughput transcriptome analysis by Next Generation Sequencing, specifically RNAseq, is currently widely available. As shown in the case of rice miRNAs, these techniques may help answer the question about the novel small RNAs recently discovered to be secreted.

FUTURE DIRECTIONS FOR RESEARCH ON SECRETORY RNAs

In this review, we summarized the recent findings of secretory miRNAs. The research field of secretory miRNAs has just begun. To use the knowledge of secretory miRNAs for human health, we should unveil the mystery of secretory RNA as follows.

First, we need to know the all kinds of secretory RNA species. Interestingly, Dinger et al. (2008) independently analyzed the microarray dataset from Valadi et al.'s (2007) study and found that many longer non-coding RNAs (ncRNAs) were also present in exosomes, including a number of ncRNAs associated with important genes and several known ncRNAs, such as Copg2as and Nespas, in mast cell-derived exosomes. This question seems quite easy to obtain the answer. As we already mentioned above, recent development of next generation sequencing technologies has been developed. This means that we can directly recognize the nucleic acids that can exist outer space of the cells.

Second, secretory machinery of miRNAs and other types of RNA should be clarified. As described in this paper, we recently detected the part of miRNAs secretion mechanism mediated by exosome (Kosaka et al., 2010b). Analyzing the secretion mechanism of various kinds of RNAs and sorting mechanism of miRNA into the vesicles leads the development of novel nucleic acids based medicine.

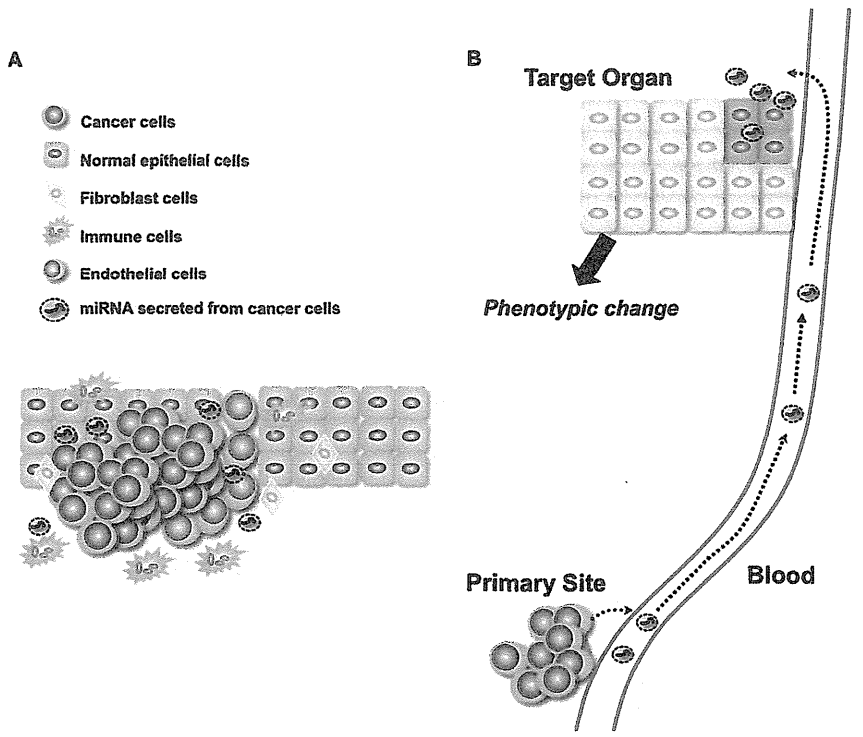

FIGURE 2 | Two proposed hypotheses regarding secretory miRNA-mediated cancer progression. (A) Secretory miRNAs mediate the modulation of microenvironmental cells. Secretory miRNAs from cancer cells control the behavior of microenvironmental cells for their own benefit. **(B)** Secretory miRNAs could be functional molecules in a distant organ.

Last point is to know the function of secretory miRNAs in more detail, such as physiological conditions and pathological conditions. Reports on the function of secretory miRNAs in physiological conditions, such as embryogenesis, organogenesis, and maintaining tissue and organ homeostasis, are not available. In addition, to know the function of secretory miRNAs, we need to know the incorporation mechanism of miRNAs. Although SID-1 found in *C. elegans* is good example of secretory miRNA transport mechanism, the other machinery might exist in vertebrates. Indeed, both transporter-dependent (SID-1 dependent) and transporter-independent (SID-1 independent) dsRNA export takes place from *C. elegans* cells (Jose et al., 2009). Furthermore, as shown previously, miRNA from plant was detected in human circulating peripheral blood, and the function of plant derived miRNA was documented by the authors, suggesting that other types of small RNA from other species might contribute to the regulating of physiological or pathological situation. Because miRNAs act as multi-functional molecules via the binding to sequence similarities and regulate various life phenomena, secretory miRNAs might be a humoral factor that exerts its influence in distant organs, similarly to hormones. Clarifying the species, mechanisms and roles of secretory miRNA, and other secretory ncRNAs in both pathological and physiological conditions would unveil the mystery of "secretory miRNAs-mediated disease" (**Figure 1**).

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Inhibition of Stabilin-2 elevates circulating hyaluronic acid levels and prevents tumor metastasis

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Hyaluronic acid (HA) has been implicated in the proliferation and metastasis of tumor cells. However, most previous studies were conducted on extracellular matrix or pericellular HA, and the role of circulating HA in vivo has not been studied. HA is rapidly cleared from the bloodstream. The scavenger receptor Stabilin-2 (Stab2) is considered a major clearance receptor for HA. Here we report a dramatic elevation in circulating HA levels in Stab2-deficient mice without any overt phenotype. Surprisingly, the metastasis of B16F10 melanoma cells to the lungs was markedly suppressed in the Stab2-deficient mice, whereas cell proliferation was not affected. Furthermore, administration of an anti-Stab2 antibody in Stab2$^+$ mice elevated serum HA levels and prevented the metastasis of melanoma to the lung, and also suppressed spontaneous metastasis of mammary tumor and human breast tumor cells inoculated in the mammary gland. Administration of the antibody or high-dose HA in mice blocked the lodging of melanoma cells to the lungs. Furthermore, HA at high concentrations inhibited the rolling/tethering of B16 cells to lung endothelial cells. These results suggest that blocking Stab2 function prevents tumor metastasis by elevating circulating HA levels. Stab2 may be a potential target in antitumor therapy.

cancer | hyaluronan | imaging | antibody therapy | sinusoid

Scavenger receptors mediate the endocytosis of metabolic waste products produced under normal and pathological conditions, as well as harmful foreign substances, such as bacterial debris absorbed in the gut. The liver functions as a major filter to eliminate such molecules from the circulation. Liver-specific capillaries known as sinusoids are vital to this function; for example, more than 90% of circulating hyaluronic acid (HA) is cleared by liver sinusoids (1). Sinusoidal walls consist of hepatic sinusoid endothelial cells (HSECs), stellate cells, and liver resident macrophages known as Kupffer cells. HSECs and Kupffer cells express various types of scavenger receptors to fulfill the filter functions. Among those scavenger receptors, Stabilin-1 (Stab1, also known as FEEL-1 and CLEVER-1) and Stabilin-2 (Stab2, also known as FEEL-2 and HARE) are structurally related, exhibiting 55% homology at the protein level, and expressed on HSECs (2).

Stab1 and Stab2 are large type I transmembrane glycoproteins containing four domains with EGF-like repeats, seven fasciclin-1 domains, and an X-link domain (3). Despite these two glycoproteins' structural similarity, the spectrum of their ligands differs significantly. Stab1 is expressed on lymphatic vessels and macrophages as well as HSEC and binds to acetylated low-density lipoprotein (ac-LDL), secreted protein acidic and rich in cysteine, placental lactogen, growth differentiation factor 15, and Gram-positive and Gram-negative bacteria, but not to HA (2, 4–8). It also mediates leukocyte trafficking (9). Stab2 is expressed on the sinusoid endothelium in the liver, spleen, and lymph nodes and has been used as a specific marker for HSECs (10). It binds to and mediates the endocytosis of HA, advanced glycation end products-modified protein, and heparin in addition to ac-LDL, growth differentiation factor 15, and bacteria (2, 4). Stab2 also recognizes membrane phosphatidylserine of apoptotic cells (11). Previous studies found that unlabeled chondroitin sulfate inhibited the uptake of ^{125}-I-HA (12), and that ac-LDL binding to Stab2 was partially competed by heparin and dextran sulfate, but not competed by HA (13). These findings suggest that the HA binding site overlaps with the binding site of chondroitin sulfate but differs from the binding sites of ac-LDL, heparin, and dextran sulfate.

HA is a glycosaminoglycan of the extracellular matrix consisting of tandem repeats of D-glucuronic acid and *N*-acety-D-glucosamine. HA is abundant in the umbilical cord, articular joints, cartilage, and vitreous humor (14). It has been implicated in various physiological functions, including lubrication, water homeostasis, filtering effects, regulation of plasma protein distribution, angiogenesis, wound healing, and chondrogenesis (15). Signal transduction and functions of HA differ depending on molecular size; for example, high molecular weight HA suppresses angiogenesis, whereas HA fragments stimulate angiogenesis (16).

HA interacts with various cell surface receptors, including CD44, Lyve-1, TLRs, RHAMM, and Stab2 (17, 18). CD44, the most extensively characterized of these receptors, is expressed at varying levels in most immune cells and is involved in their rolling and extravasation via HA displayed on endothelial cells (ECs) (19). CD44 is also implicated in tumorigenesis and a marker for cancer stem cells (reviewed in ref. 20). Lyve-1 is structurally related to CD44 and is expressed in lymphatic vessels as well as in HSECs (21). TLR2 and TLR4 bind to HA or a complex of HA and HA-binding protein (18, 22); however, none of the mice deficient for CD44, Lyve-1, or TLRs have been shown to affect circulating HA levels in vivo. Although Stab1 and Stab2 are structurally related scavenger receptors with the HA-binding link domain, only Stab2 binds HA, and thus it has been considered the primary scanvenger receptor for HA (2, 3, 5).

HA, HA synthases (HAS), hyaluronidases, and HA receptors have been implicated in various tumors, including carcinomas, lymphomas, and melanocytic and neuronal tumors (23, 24). Overexpression and knockdown of HAS and hyaluronidases

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