

novel genetic exchange between cells using miRNA either in microvesicles (up to 1  $\mu\text{m}$ ) or in small membrane vesicles of endocytic origin called exosomes (50–100 nm) (Table 2).<sup>(23–32)</sup> One of the first reports showing the existence of miRNA in exosomes was studied by Valadi *et al.*<sup>(32)</sup> who reported that exosomes released from human and murine mast cell lines contain mRNAs and miRNAs. Hunter *et al.*<sup>(33)</sup> demonstrated that miRNAs contained in the microvesicles from blood were known to regulate the cellular differentiation of blood cells and metabolic pathways and to modulate immune function. On the other hand, evidence of the presence of tumor-derived exosomes in the peripheral circulation was provided by Taylor *et al.*<sup>(9)</sup> Furthermore, they compared tumor-derived miRNA profiles and peripheral blood-derived exosomal miRNAs and showed that they were not significantly different. In addition, Rabinowits *et al.*<sup>(34)</sup> reported that the protein concentration of circulating exosomes was significantly higher in lung adenocarcinoma than in a control group. Significant differences were also found between the mean exosomal miRNA concentrations of the lung adenocarcinoma group and the control group. Interestingly, in four cases of lung adenocarcinoma in which paired tumor and plasma samples were examined, there was a close correlation between the circulating miRNAs of tumor-derived exosomes and tumor miRNAs. Skog *et al.*<sup>(26)</sup> demonstrated that brain microvascular endothelial cells take up exosomes, which contain mRNA, miRNA, and angiogenic proteins released by glioblastoma cells. Moreover, miR-21, known to be overexpressed in glioblastoma tumors, was more elevated in serum microvesicles from glioblastoma patients than in healthy controls.

Considering that exosomes and microvesicles are evident in several types of body fluid from cancer patients, miRNA surely be able to be found not only in serum/plasma but also in other body fluid. Indeed, Michael *et al.*<sup>(35)</sup> showed the presence of miRNAs within exosomes isolated from human saliva. Furthermore, Park *et al.*<sup>(36)</sup> found that miR-125a and miR-200a were present in significantly lower levels in the saliva of oral SCC patients than in control subjects. Recently, we found miRNA presence in human breast milk and detected high expression levels of immune-related miRNAs in the first 6 months of lactation.<sup>(37)</sup> It is noteworthy that these miRNA molecules are stable even in very acidic conditions, indicating that breast milk allows the dietary intake of miRNAs by infants. Thus, these reports raise the possibility that circulating miRNAs could be used as non-invasive diagnostic markers.

## Mechanism of miRNA secretion and incorporation

We have summarized recent reports that show the existence of circulating miRNAs in the blood of cancer patients (Fig. 1). To use circulating miRNAs as a diagnostic marker, we need to gain a better understanding of the mechanisms by which miRNAs are released in the bloodstream. In some organisms, such as *C. elegans* and *Drosophila melanogaster*, small RNA pathways can spread from cell to cell. However, the secretory mechanism and incorporation of extracellular miRNAs in mammalian cells remain unclear.

Rechavi *et al.*<sup>(38)</sup> showed that T cells receive small RNAs from B cells that can affect the expression of target genes in the recipient T cells upon cell contact. Furthermore, Pegtel *et al.*<sup>(39)</sup> demonstrated that miRNAs secreted by Epstein–Barr virus (EBV)-infected cells are transferred to and act in uninfected recipient cells through exosomes. They also showed that these EBV-miRNAs repressed confirmed EBV target genes. Importantly, although EBV DNA is restricted to the circulating B-cell population, Pegtel *et al.* also showed that EBV BamHI-A Rightward Transcript (BART) miRNAs are present in both B-cell and non-B-cell fractions in peripheral blood mononuclear cells from patients with increased EBV. In addition, Yuan *et al.*<sup>(40)</sup> showed that embryonic stem (ES) cell microvesicles contain abundant miRNA and that they can transfer a subset of miRNAs to mouse embryonic fibroblasts *in vitro*, suggesting that stem cells can affect the expression of genes in neighboring cells by transferring miRNAs contained in microvesicles. As in these reports, several other studies have already shown the intercellular transfer of miRNAs from donor cells to recipient cells; however, there are only a few reports showing the mechanism of secretion of miRNAs. Recently, we showed that miRNAs are released through a ceramide-dependent secretory machinery and the secretory miRNAs are transferable and functional in the recipient cells.<sup>(41)</sup> We clarified that neutral sphingomyelinase 2 (nSMase2), which regulates ceramide biosynthesis, controls the secretion of miRNAs outside the cells. This enzyme is already known for the secretion of exosomes. However, the endosomal sorting complex required for the transport (ESCRT) system, which is another regulator of exosome secretion, is unnecessary for the release of miRNAs. Furthermore, a tumor-suppressive miRNA secreted via a ceramide-dependent pathway suppressed its target gene in the recipient cells, thereby leading to cell growth inhibition. In this report, we showed the importance of nSMase2 in the secretion of miRNA; however, there might be

**Table 2. Particles in body fluid and their roles in recipient cells**

Particles	Typical size	Origin of particle	Reported function of particles to recipient cells	Reference
Microvesicle	0.1–1 $\mu\text{m}$	BCLL	Stimulate bone marrow stromal cells to induce the production of VEGF	24
		Melanoma cells	Increased TGF- $\beta$ 1 production by cultured macrophages Enhanced the metastatic potential of melanoma cell lines <i>in vivo</i>	25
Exosome	10–100 nm	Glioblastoma cells	Stimulate tubule formation by endothelial cells	26
		Mammary adenocarcinoma cells	Induce potent CD8+ T-cell-dependent antitumor effects by dendritic cells	27
Prostasome	50–500 nm	Prostatic ductal epithelial cells	Boosting survivability, motility of spermatozoa and modulate acrosomal reactivity	28
		Prostate cancer cells	Protect prostatic malignant cells from complement attack	29
Apoptotic body	0.5–2 $\mu\text{m}$	EBV-carrying Burkitt's lymphoma cell line	Induce the expression of EBV-specific markers in the recipient cells	30
		Endothelial cells	Induce the expression of CXCL12 in endothelial cells	31

BCLL, B-cell chronic lymphocytic leukemia; CXCL12, chemokine CXC motif ligand 12; EBV, Epstein–Barr virus; TGF- $\beta$ 1, transforming growth factor  $\beta$ 1; VEGF, vascular endothelial growth factor.

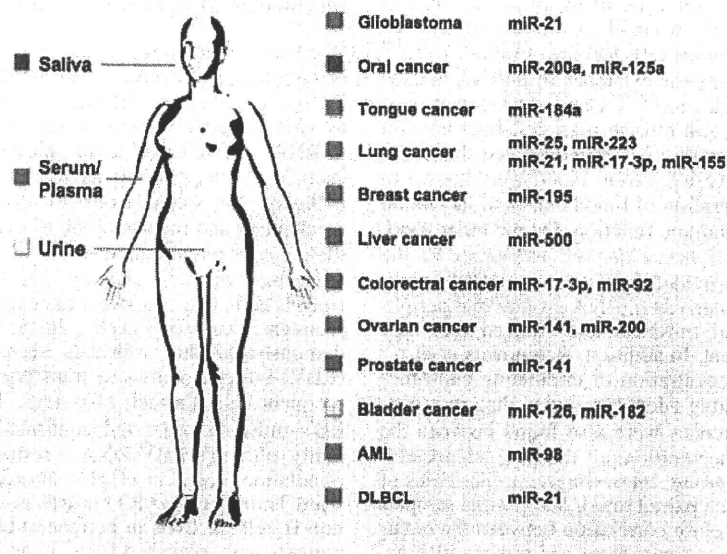


Fig. 1. miRNAs in human body fluids are non-invasive diagnostic markers for cancers. Many kinds of circulating miRNAs have been reported in various types of cancers. However, certain cancers cannot be diagnosed by known serum biomarkers. In such cases, circulating miRNAs in serum, saliva, and urine are good candidates for future use. AML, acute myeloid leukemia; DLBCL, diffuse large B-cell lymphoma.

another mechanism for the secretion of miRNAs from cells. One candidate is an apoptotic body, namely, the membranous microvesicles shed from cells during apoptosis. Zernecke *et al.*<sup>(31)</sup> showed that endothelial cell-derived apoptotic bodies are generated during atherosclerosis and carry paracrine alarm signals to recipient vascular cells, which trigger the production of chemokine CXCL12. CXCL12 production was mediated by miR-126. miR-126 was enriched in apoptotic bodies and repressed its target gene in recipient cells. This study showed an important observation, namely, that not only exosomes but also apoptotic bodies can transfer miRNAs between the cells. Interestingly, a recent report showed the relationship between an exosome and an apoptotic body. Phosphatidylserine, which is exposed on the surface of apoptotic cells and works as an “eat me” signal for phagocytes, is also expressed on the surface of exosomes. Miyanishi *et al.*<sup>(42)</sup> found an antibody that inhibited the phosphatidylserine-dependent engulfment of apoptotic cells, and the antigen recognized by the antibody was identified by expression cloning as a type I transmembrane protein called Tim4 (T-cell immunoglobulin- and mucin-domain-containing molecule). The expression of Tim4 in fibroblasts enhanced their ability to engulf apoptotic cells, and Tim4-expressing cell lines were bound by exosomes via phosphatidylserine. These results indicate that Tim4 is a phosphatidylserine receptor for the engulfment of apoptotic cells and may also be involved in exosome-mediated intercellular signaling.

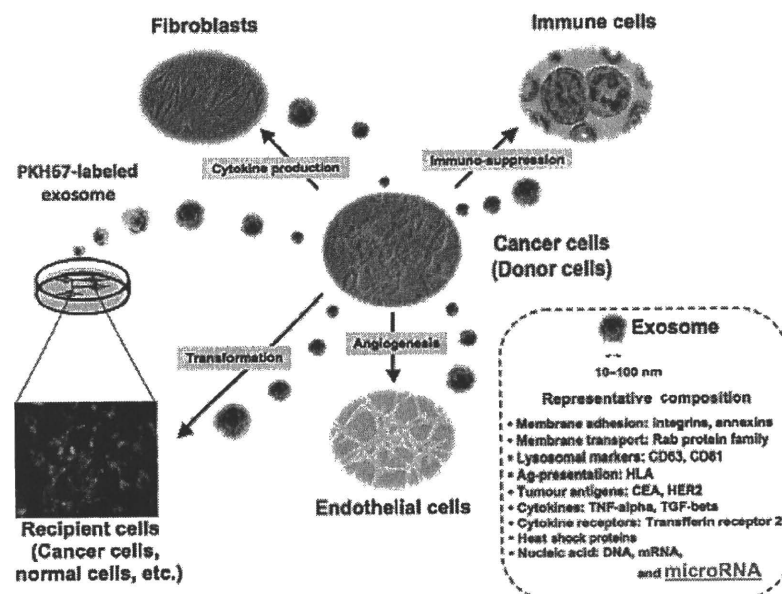
As reported above, RNA interference (RNAi), which silences genes of a matching sequence by double-strand RNA (dsRNA), spreads systemically in plants and nematodes to silence gene expression distant from the site of initiation. Passive dsRNA uptake has been uniquely observed in *C. elegans* due to the expression of systemic RNA interference defective-1 (SID-1). Tsang *et al.*<sup>(43)</sup> showed that ectopic expression of SID-1 induced the cellular uptake of siRNA or dsRNA in mouse ES cells. The mammalian SID-1 homologue localizes to the cell membrane of human cells and enhances their uptake of siRNA, resulting in an increased siRNA-mediated gene silencing effect. Recently, Wolfrum *et al.*<sup>(44)</sup> reported that cholesterol-conjugated siRNAs could silence gene expression *in vivo*. These reports clarify that

not only are the low-density lipoprotein (LDL)- and scavenger receptor class B type I (SR-BI) receptors essential for siRNA delivery by LDL particles and high-density lipoprotein (HDL)-bound siRNAs but also SID-1 is required for cellular uptake. There are still no answers with regard to the uptake of miRNA from donor cells; however, we might have a tightly regulated mechanism for the control of miRNA incorporation.

The phenomenon of secretion and incorporation of miRNAs seems to be a general biological event for organisms. Interestingly, recent studies have shown the importance of communication between cancer cells and their surroundings through exosomes (Fig. 2). For instance, epidermal growth factor receptor variant III (EGFR vIII) proteins were transferred into glioma cells lacking EGFRvIII via secretory membrane microvesicles.<sup>(45)</sup> Because many tumors have a remarkable ability to mold their stromal environment to their own advantage, exosomes of cancer cells can contribute to the horizontal propagation of oncogenic miRNAs and their associated transforming phenotype among subsets of cancer cells. Although the mechanism of secretion and incorporation of miRNAs has not been clarified, secretory miRNAs may play a pivotal and general role as a signaling molecule in physiological and pathological events.

## Discussion

Since cancer is fundamentally a dysregulation of gene expression, it is difficult to distinguish tumors which are morphologically similar but molecularly different by pathological assessment. For the earliest diagnosis, it is necessary to find non-invasive cancer biomarkers to monitor molecular differences in tumors, which may assist in the selection of the best possible treatment for individual cancer patients. These cancer biomarkers include carcinoembryonic antigen (CEA), which is a commonly used marker of colon cancer, alpha-fetoprotein (AFP), which is an associated marker for HCC, and the prostate-specific antigen (PSA), a protein normally present at low levels in the blood of adult men that has a high association with prostate cancer. However, these cancer biomarkers are also elevated



**Fig. 2.** Secreted miRNAs contained in exosomes potentially influence microenvironmental cells, including immune cells, endothelial cells, and fibroblast cells. Soluble factors, such as cytokines and chemokines, have been shown to be intercellular communication tools between cancer and microenvironmental cells. In addition, recent observations have indicated that exosomes also function as an intercellular communication tool between them. For instance, exosomes, which are secreted by living tumor cells, contain and transfer tumor antigens to dendritic cells, and dendritic cells then induce potent CD8+ T-cell-dependent antitumor effects on syngenic and allogeneic established mouse tumors. In addition, this transforming activity can possibly be transferred via exosome. Furthermore, miRNA, which regulates multiple target genes, can also be transferred from tumor cells to tumor cells or tumor cells to normal cells. When purified PKH67-labeled exosomes from donor cancer cells were incubated with recipient cancer cells, they became fluorescent, indicating that an oncogene can be propagated horizontally through exosomes. CEA, carcinoembryonic antigen; HLA, human leukocyte antigen; HER2, human epidermal growth factor receptor 2; TGF; transforming growth factor; TNF, tumor necrosis factor.

in benign conditions. For instance, prostate inflammation and benign prostatic hypertrophy result in increased PSA blood levels. Furthermore, blood levels of CEA and AFP are also elevated in benign diseases, such as cirrhosis, inflammatory bowel disorder, chronic lung disease and pancreatitis (CEA), and hepatitis and cirrhosis (AFP). Several current reports suggest that deregulation of miRNAs is tightly linked to cancer incidence and, in particular, that some miRNAs are closely associated with clinical prognosis. Therefore, it is anticipated that circulating miRNAs in plasma and/or serum may become novel methods for reducing both false-positive and false-negative results when undertaken by the conventional diagnostic method. Although the analysis of circulating miRNAs has just begun, the indications that such circulating miRNAs may have a biological role and may be involved in transforming cells suggest that these miRNAs may have potential as diagnostic, prognostic, and predictive biomarkers and may also be considered as therapeutic targets.

In this review, we have shown that circulating miRNAs are promising biomarkers for cancer diagnosis and prognosis; however, there have been conflicting findings about circulating miRNAs from the same tumor reported from different studies (Table 1). This might be due to the lack of an established endogenous miRNA control to normalize for circulating miRNA levels. In addition, a different extraction and quantification method among the studies also produced conflicting results. It is

very difficult to determine which endogenous control is suitable for measuring circulating miRNAs because the expression profile of circulating RNA may change depending on the condition of the cancer patient, such as, for example, whether the patient is awaiting treatment, receiving chemotherapy, or post surgery. According to this view, it would be necessary to perform a well-controlled analysis of circulating miRNAs in a large cohort of patients and healthy volunteers. These studies will provide further evidence in which miRNAs may be useful as serum biomarkers in clinical usage.

All the findings discussed in this report suggest that circulating miRNAs are promising as novel non-invasive biomarkers useful for the elimination of false positives and false negatives of conventional various classifiers; however, the function of circulating miRNAs needs to be identified for the proper use of circulating miRNA biomarkers in evidence-based medicine.

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# Secretory Mechanisms and Intercellular Transfer of MicroRNAs in Living Cells<sup>\*S</sup>†

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The existence of circulating microRNAs (miRNAs) in the blood of cancer patients has raised the possibility that miRNAs may serve as a novel diagnostic marker. However, the secretory mechanism and biological function of extracellular miRNAs remain unclear. Here, we show that miRNAs are released through a ceramide-dependent secretory machinery and that the secretory miRNAs are transferable and functional in the recipient cells. Ceramide, whose biosynthesis is regulated by neutral sphingomyelinase 2 (nSMase2), triggers secretion of small membrane vesicles called exosomes. The decreased activity of nSMase2 with a chemical inhibitor, GW4869, and a specific small interfering RNA resulted in the reduced secretion of miRNAs. Complementarily, overexpression of nSMase2 increased extracellular amounts of miRNAs. We also revealed that the endosomal sorting complex required for transport system is unnecessary for the release of miRNAs. Furthermore, a tumor-suppressive miRNA secreted via this pathway was transported between cells and exerted gene silencing in the recipient cells, thereby leading to cell growth inhibition. Our findings shed a ray of light on the physiological relevance of secretory miRNAs.

It has been well known that extracellular RNAs circulate in the blood of healthy people and diseased patients with sufficient integrity, although ribonuclease is present in both plasma and serum (1). The spectrum of RNAs whose presence was demonstrated in plasma and other body fluids, such as urine and breast milk, extends from housekeeping genes to fetal genes detected in pregnant women and genes overexpressed in a variety of different tumors (2). To explain the stability of circulating RNAs, it was suggested that extracellular RNAs are included within lipoprotein vesicles. Indeed, exogenous RNAs added to plasma or blood are immediately degraded, whereas endoge-

nous plasma RNAs are stable for hours under the same conditions (3). Moreover, the treatment of some detergents results in immediate degradation of plasma extracellular RNAs, apparently due to disruption of the lipid vesicles. These findings clearly indicate that extracellular RNAs are packaged in some kinds of secretory particles including apoptotic bodies and exosomes, and thus, they are protected from dominantly existing ribonucleases.

Apoptotic bodies are small membranous particles released during programmed cell death (4), and exosomes are small intraluminal vesicles (50–100 nm in diameter) of multivesicular bodies (MVB)<sup>3</sup> released on exocytic fusion of MVB with plasma membranes (5). Currently, accumulating evidence suggests that these secretory vesicles can function as intercellular transmitters to convey their contents, in particular, microRNA (miRNA) (6–8). Recent studies reported that extracellular exosomal miRNAs were transferred into other cells and that apoptotic bodies delivered miR-126 into endothelial cells (9). Despite these advances, however, the underlying mechanism of the secretory process and the biological function of circulating miRNAs are not yet fully understood.

miRNAs, small 20–22-nucleotide-long members of the non-protein-coding RNA family, are expressed in the vast majority of eukaryotes, including humans (10). Not only do they inhibit translation of their target genes, they also degrade the target mRNAs through recognition of imperfect complementary sites, usually located in the 3′-untranslated regions of the target mRNAs, endowing miRNAs with the capacity to regulate numerous biological processes. Over the past several years, it was evident that dysregulations of many kinds of miRNAs have been linked to the initiation and progression of human cancer (11). Interestingly, the amounts of secretory miRNAs are up-regulated in the plasma of patients bearing tumors, including B cell lymphoma, prostate cancer, lung cancer, and ovarian cancer (12–15). Thus, detection and monitoring of tumors are now becoming possible by the evaluation of tumor-derived secretory miRNAs.

In this study, we have shown that secretion of miRNAs is controlled by neutral sphingomyelinase 2 (nSMase2), which is known as a rate-limiting enzyme of ceramide biosynthesis. Fur-

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<sup>3</sup> The abbreviations used are: MVB, multivesicular bodies; Alix, ALG-2 interacting protein; ESCRT, endosomal sorting complex required for transport; miRNA, microRNA; nSMase2, neutral sphingomyelinase 2; siRNA, small interfering RNA; QRT-PCR, quantitative real time PCR; CM, conditioned medium.

thermore, we provide evidences that miRNAs secreted from donor cells can be taken up and function in the recipient cells. These findings propose a novel mechanism of intercellular communication mediated by secretory miRNAs.

## EXPERIMENTAL PROCEDURES

**Reagents**—Rabbit polyclonal anti-nSMase2 (H-195) (sc-67305), goat polyclonal anti-Alix (Q-19) (sc-49268), and donkey anti-goat IgG (horseradish peroxidase) (sc-2020) were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-ROCK1 (ab36746) was from Abcam. Mouse monoclonal anti-actin, clone C4 (MAB1501), was from Millipore. Anti-CD63 monoclonal antibody 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-146a (pre-miR-146a) was purchased from Ambion (Austin, TX). The duplexes of each small interfering RNA (siRNA), targeting human nSMase2 mRNA (s30925; target sequences of 5'-GGAGGUGUUUGACAAGCGAdTdT-3' and 5'-UCGCUUGUCAACACCCUCctg-3'), and negative control 1 (NC1) were purchased from Applied Biosystems, and an siRNA specific for human ALG-2 interacting protein (Alix) mRNA (target sequences of 5'-GAACCUGGAUAAUGAUGAAdTdT-3' and 5'-UUCAUCAUUAUCCAGGUUCdTdT-3') was purchased from Sigma-Genosys. GW4869 was purchased from Calbiochem (Darmstadt, Germany). Cisplatin was obtained from Alexis (Lausen, Switzerland). Geneticin was purchased from Invitrogen.

**Cell Culture**—HEK293 cells, a human embryonic kidney cell line (CRL-1573), and COS-7 cells, an African green monkey kidney fibroblast-like cell line (CRL-1651), were obtained from the American Type Culture Collection (Manassas, VA). These cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum and antibiotic-antimycotic (Invitrogen) at 37 °C in 5% CO<sub>2</sub>. PC-3M-luc cells (Xenogen) were cultured in RPMI containing 10% heat-inactivated fetal bovine serum and antibiotic-antimycotic at 37 °C in 5% CO<sub>2</sub>.

**Preparation of Conditioned Medium and Exosome**—Prior to collection of culture medium, HEK293 and COS-7 cells were washed three times with Advanced RPMI containing antibiotic-antimycotic and 2 mM L-glutamine (medium A), and the medium was switched to fresh medium A. After incubation during 3 days, medium A was collected and centrifuged at 2,000 × g for 15 min at room temperature. To thoroughly remove cellular debris, the supernatant was centrifuged again at 12,000 × g for 35 min at room temperature. Then the conditioned medium was used for miRNA extraction and functional assays as well as exosome isolation.

For exosome preparation, the conditioned medium was ultracentrifuged at 110,000 × g for 70 min at 4 °C. The pellets were washed with 11 ml of phosphate-buffered saline, and after ultracentrifugation, they were resuspended in phosphate-buffered saline. The exosome fraction was measured for its protein

content using the Micro BCA protein assay kit (Thermo Scientific).

**Isolation of MicroRNAs**—Isolation of extracellular and cellular miRNAs was performed using the mirVana isolation kit (Ambion). One hundred μl of conditioned medium or cell lysate was diluted with 200 μl of lysis/binding solution. After a 5-min incubation, 20 μl of miRNA homogenate additive and 1 μl of 1 nM cel-miR-39 were added to each aliquot, followed by vortex for 30 s and incubation on ice for 10 min. Subsequent phenol extraction and filter cartridge work were carried out according to the manufacturer's protocol.

**RNA Detection**—Detection of RNAs was performed by using the Agilent Bioanalyzer 2100 (Agilent Technologies). Prior to the analysis, total RNAs were prepared with the Agilent RNA 6000 Pico kit (Agilent Technologies) according to the manufacturer's protocol.

**RNase Treatment**—To evaluate whether small RNAs were present inside the exosomes, RNase mixture (Ambion) was added to conditioned medium at a final concentration of 5 units/ml RNase A and 200 units/ml RNase T1 and then incubated at 37 °C for 30 min. Small RNAs were purified using the mirVana miRNA isolation kit (Ambion) as described above.

**Quantitative Real Time PCR (QRT-PCR)**—The method for QRT-PCR has been previously described (13). PCR was carried out in 96-well plates using the 7300 Real Time PCR System (Applied Biosystems). All reactions were done in triplicate. All of the TaqMan microRNA assays were purchased from Applied Biosystems. cel-miR-39 and hRNU6 were used as invariant control for conditioned medium and cell, respectively. The concentrations of extracellular miRNAs were calculated based on their C<sub>t</sub> values normalized by those of cel-miR-39, which was spiked in each aliquot of QRT-PCR reaction at 1 nM.

**Immunoprecipitation**—Conditioned medium was incubated with magnetic beads (Invitrogen) coated with purified anti-CD63 antibody or mouse IgG1 control antibody (Millipore) at 4 °C overnight. After washing with phosphate-buffered saline, miRNAs were extracted and applied to QRT-PCR.

**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-CD63 (1:100), anti-nSMase2 (1:200), anti-Alix (1:100), anti-ROCK1 (1:200), and anti-actin (1:1,000) 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 each 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.).

**Plasmids**—psiRNA-LucGL3 was purchased from InvivoGen. Primary-miR-143 and primary-miR-146a expression vectors were purchased from TaKaRa BIO. A full-length human nSMase2 cDNA was cloned into pIRES2-EGFP vector (Clontech). Primary miRNAs (pri-miR-155, pri-miR-16, and pri-miR-21) were PCR-amplified from human genomic DNA and cloned into the downstream of cytomegalovirus promoter in pIRES2-EGFP. For luciferase-based reporter gene assays, pLuc-Neo was constructed by inserting a firefly luciferase gene

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derived from pGL3-control (Promega) into pEYFP-1 vector (Clontech) at the BglII and AflIII sites. Sensor vector for miR-146a was constructed by introducing tandem binding sites with perfect complementarity to miR-146a, separated by a four-nucleotide spacer into the NotI site of psiCHECK2 (Promega). The sequences of the binding site are as follows: 5'-AAACCT-AGAGCGGCCGCAACCCATGGAATTCAGTTCTCAA-GAATTCCTAACCCATGGAATTCAGTTCTCAGCGGC-CGCTGGCCGCAA-3' (sense) and 5'-TTGCGGCCAGCG-GCCGCTGAGAACTGAATTCATGGGTTAAGAATTC-TTGAGAACTGAATTCATGGGTTGCGGCCGCTCT-AGGTTT-3' (antisense). The "seed" sequence of miR-146a is indicated in bold and italics. In a mutated miR-146a sensor vector, the seed sequence, AGTTCTCA, was displaced with CTGGAGAC. All the plasmids were verified by DNA sequencing.

**Transient Transfection Assays**—Plasmid transfections to HEK293 and COS-7 cells were performed using Lipofectamine LTX (Invitrogen). Cell numbers and amounts of plasmids for each transfection were determined according to the manufacturer's protocol.

Transfections of siRNA and miRNA were accomplished with DharmaFECT transfection reagent (Thermo Scientific) according to the manufacturer's protocol. The total amounts of small RNAs for each transfection were equally adjusted by the addition of NC1.

**Establishment of Stable Cell Lines**—Stable HEK293 cell lines expressing firefly luciferase or miR-146a were generated by selection with 300  $\mu\text{g}/\text{ml}$  Geneticin. HEK293 cells were transfected with 0.5  $\mu\text{g}$  of pLucNeo vector or pri-miR-146a expression vector at 90% confluency in 24-well dishes using a Lipofectamine LTX reagent in accordance with the manufacturer's instructions. Twelve h after the transfection, the cells were replated 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 were then cultured for another 2 weeks. The cells expressing the largest amount of firefly luciferase or miR-146a among transfectants were used as luciferase stably expressing cells and miR-146a stably expressing cells, respectively.

**Luciferase Reporter Assay**—HEK293 and COS-7 cells were cultured at a density of  $5 \times 10^4$  and  $1 \times 10^4$  cells/well, respectively, in 96-well tissue culture plates overnight, and miRNA transfections or the addition of conditioned medium were performed as described under the legend for Figs. 5–7. The cells were harvested, and *Renilla* luciferase activity was measured and normalized by firefly luciferase activity (16). All assays were performed in triplicate and repeated at least three times, and the most representative results are shown.

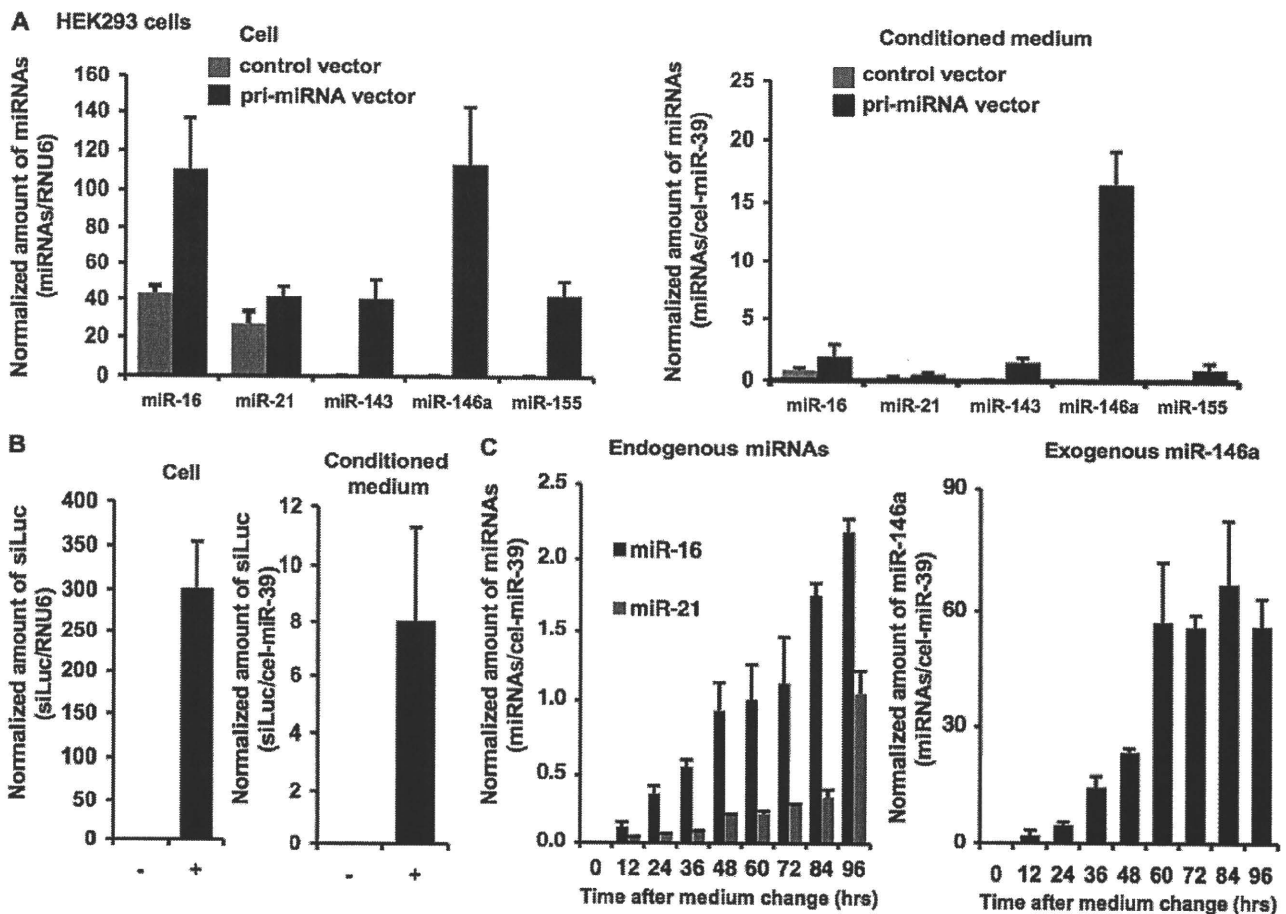
**Cell Growth Assay**—PC-3M-luc cells were seeded at a density of  $2 \times 10^3$  cells/well in a 96-well plate. The following day, the cells were transfected with mature miRNAs or were incubated with a conditioned medium. Twenty-four h later, the culture medium of the transfected cells was switched 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.

**Measurement of Caspase-3/7 Activities**—On day 0, HEK293 cells were seeded at a density of  $2.5 \times 10^4$  cells/well in a 96-well tissue culture plate. The following day, the cells were transfected with nSMase2 expression vector or were treated with 10  $\mu\text{M}$  cisplatin. On day 2, the cells were applied to an Apo-ONE homogeneous caspase-3/7 assay (Promega). After a 12-h incubation, the fluorescence of each well was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm using Envision (Wallac).

## RESULTS

**Overexpression of miRNA in Cells Leads to an Increased Secretion**—To reveal the physiological roles of extracellular miRNAs, we investigated the mechanism of their secretion and whether or not secretory miRNAs can function in cells beyond their own cell origin. Based on the observation that expression patterns of miRNAs in a cell and culture medium are well correlated (7, 17), we hypothesized that up-regulation of cellular miRNAs leads to an increase of extracellular miRNAs in a conditioned medium. To test this hypothesis, we quantified the amount of extracellular and cellular miRNAs (miR-16, -21, -143, -146a, and -155) in HEK293 and COS-7 cells transfected with each primary miRNA expression vector or empty vector. We avoided the use of synthetic analogues of mature miRNAs for this overexpression experiment because they might persist in the medium and interfere with accurate quantification of extracellular miRNAs. The transfected cells were thoroughly washed prior to the medium change to remove any surplus liposome left by the original transfection. QRT-PCR analysis showed that secretion of the miRNAs was enhanced in proportion to their cellular amounts induced by the transfections of pri-miRNA vectors (Fig. 1A; supplemental Fig. 1A). To exactly estimate what percentages of the miRNAs were excreted, we re-evaluated cellular amounts of the miRNAs by the same normalization method as the extracellular miRNAs (supplemental Fig. 1B). As a result of the calculations, the amounts of secreted miRNAs were quite lower in conditioned medium than in parental cells. The ratios of secreted miRNAs/cellular miRNAs are shown as follows: endogenous miR-16,  $1.21 \pm 0.12\%$ ; endogenous miR-21,  $1.62 \pm 0.63\%$ ; exogenous miR-143,  $2.57 \pm 1.03\%$ ; exogenous miR-146a,  $15.6 \pm 1.62\%$ ; exogenous miR-155,  $1.38 \pm 0.84\%$ . Additionally, siRNAs targeting luciferase were also released from the siRNA-overexpressing cells (Fig. 1B). We further observed that endogenous miR-16 and -21 as well as overexpressed miR-146a time-dependently accumulated in the conditioned medium during 96 h (Fig. 1C). We observed by microscopic examination that these cells did not suffer from any damage and that they maintained the original cell shape. Moreover, the treatment of an apoptotic inducer, cisplatin, did not increase the miRNA secretion (data not shown). These findings strongly suggest that living cells can actively secrete endogenous and exogenous miRNAs as well as artificial small RNAs.

**Secreted miRNAs Are Contained in Exosomes**—It was revealed that exosomes can transfer some of their contents to other cell types, and importantly, that miRNAs exist in exosomes and are protected from RNases (6). First, we isolated exosome fractions from HEK293 cells by the standard ultracentrifugation protocol.



**FIGURE 1. Characterization of extracellular microRNAs.** *A*, miRNAs were released in proportion to the cellular expression level. HEK293 cells were set up at a density of  $2.5 \times 10^5$  cells/well in a 24-well plate. The following day, the cells were transfected with  $0.5 \mu\text{g}$  of the indicated primary miRNA expression vectors or pCMV empty vector as a control as described under "Experimental Procedures." Twenty-four h later, after washing three times by Advanced RPMI containing antibiotic-antimycotic and 2 mM L-glutamine (medium A), culture medium was switched to medium A. After a 24-h incubation, preparation of conditioned medium and isolation of total RNAs were performed as described under "Experimental Procedures." Expression levels of miRNAs were analyzed using quantitative real-time PCP (QRT-PCR). *B*, siRNAs targeting luciferase gene (*siLuc*) were secreted into culture medium. HEK293 cells were set up at a density of  $2.5 \times 10^5$  cells/well in a 24-well plate. Transfection with psiRNA-LucGL3 vector (+) or control vector (-) and preparation of conditioned medium were conducted as described above. The amount of luciferase siRNAs generated from the expression vector was measured by QRT-PCR with a custom-designed TaqMan small RNA Assay (Applied Biosystems) specific for the luciferase siRNAs. *C*, time course analysis of extracellular miRNAs. Conditioned medium was collected at the indicated time and applied to quantitative miRNA RT-PCR. A-C, cel-miR-39 and hRNU6 were used as invariant control for conditioned medium and cell, respectively. Each bar is presented as mean S.E. ( $n = 3$ ).

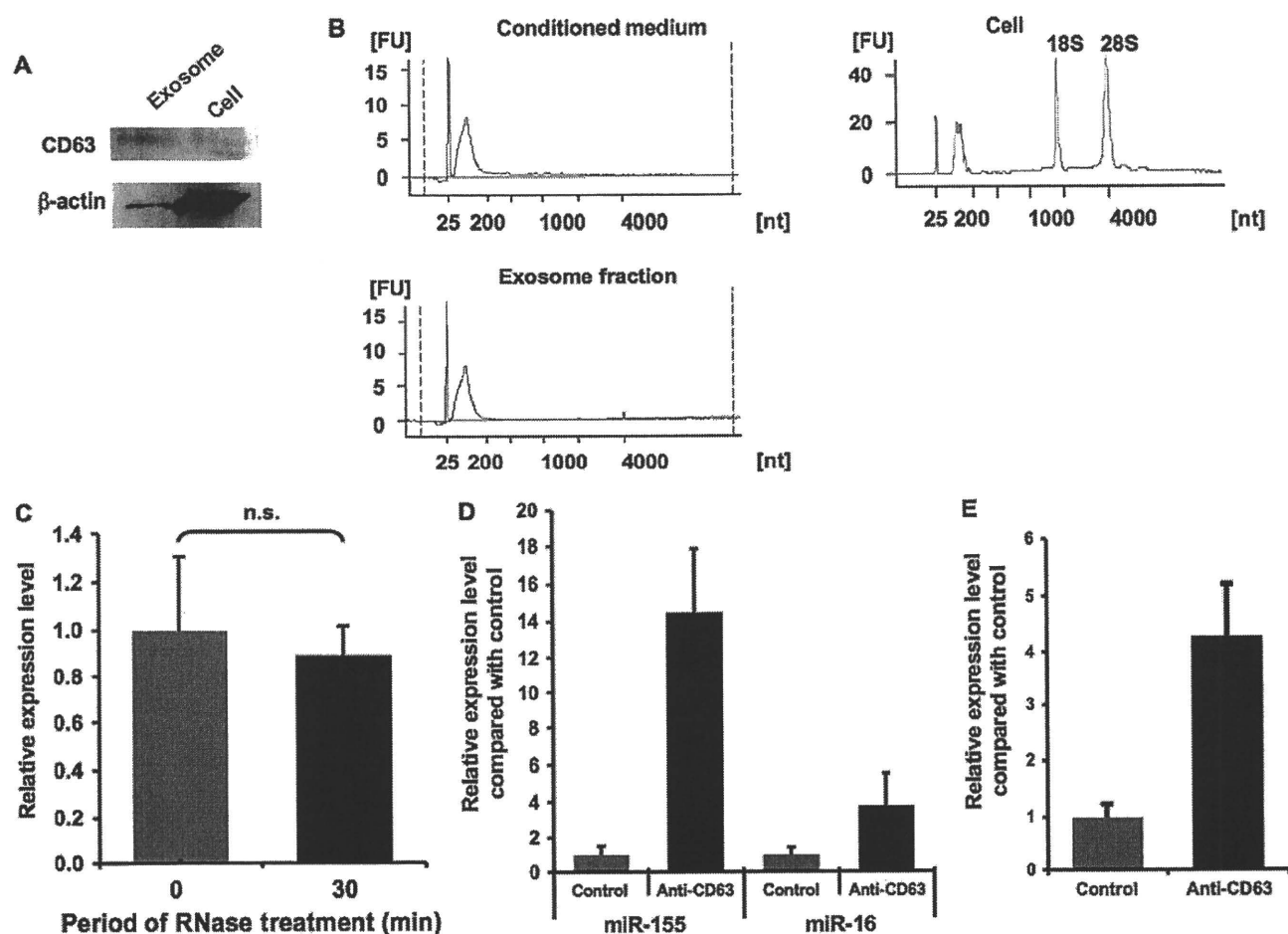
trifugation method. Consistent with the previous investigations, these exosome fractions were positive for a surface marker of exosome, CD63 (Fig. 2A). To characterize the exosomal RNAs, we conducted an electrophoresis of total RNA extracted from conditioned medium, exosomes, and their donor cells. Conditioned medium and exosomes applied to this analysis were prepared from the supernatant of the same numbers of donor cells. The Bioanalyzer 2100 profiles shown in Fig. 2B revealed that conditioned medium and exosome fractions share a very similar size-distribution pattern and expression intensity, which shows the enrichment of small sized RNAs, whereas the cellular RNA profile indicates two conspicuous peaks of 18 S and 28 S ribosomal RNAs and a broad peak of small RNAs. These data suggest that small RNAs are preferentially released from the cells and that most of the secreted RNAs are contained in exosome fractions. To evaluate whether extracellular miRNAs are contained inside exosomes, the conditioned medium from HEK293 cells was exposed to RNases

before RNA extraction. As a result, endogenous miR-21 still existed even after 30 min of treatment with RNase A and T1 (Fig. 2C). Contrarily, exogenously added synthetic cel-miR-39 was completely decomposed by the same treatment (supplemental Fig. 2). Moreover, immunoprecipitant obtained with an anti-CD63 antibody was enriched in endogenous miR-16 and -155 as well as overexpressed luciferase siRNA (Fig. 2, D and E). These results collectively indicate that extracellular miRNAs are contained within the CD63-positive exosomes and therefore are protected from external RNases by the surrounding membrane.

*The Secretion of miRNAs Is Regulated by a Ceramide-dependent Pathway*—Exosomes are produced in MVB and released from a variety of cells. They are originally thought to function to dispose of cellular garbage, including degraded protein, but now they draw much attention as a cell-cell communication tool (5). The biogenesis of exosomes has appeared to associate with the endosomal sorting complex required for transport



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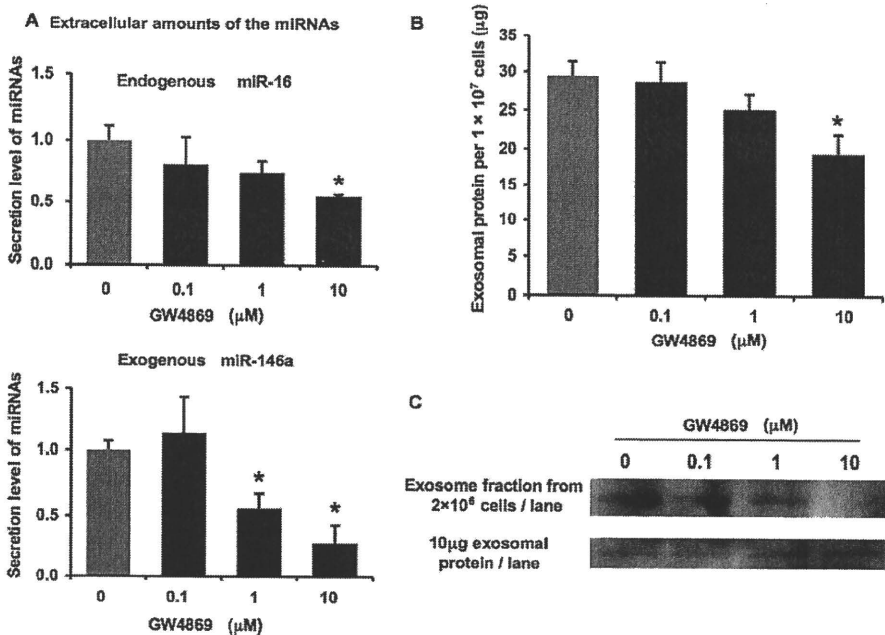


**FIGURE 2. Purification and characterization of secretory exosomes.** *A*, purified exosomes secreted from HEK293 cells are enriched in CD63 protein. The exosome fractions and whole cell lysate were analyzed by immunoblotting with an anti-CD63 antibody or anti- $\beta$ -actin antibody. *B*, total RNAs isolated from conditioned medium, exosome fractions, and their donor HEK293 cells were detected using a Bioanalyzer 2100. The obtained data show the amount and the size distribution of total RNAs. The peak migrating around 25 nucleotides (nt) represents an internal standard. FU, fluorescence units. *C*, effect of RNase treatment on extracellular miR-21. Conditioned medium from HEK293 cells was exposed to RNase A and RNase T1 at 37 °C followed by the isolation of miRNAs at the indicated times. The amount of miR-21 was determined using quantitative miRNA RT-PCR. The values on the y axis are depicted relative to the amount of miR-21 at 0 min, which is arbitrarily defined as 1. *D* and *E*, CD63<sup>+</sup> exosome fraction is enriched in miR-155 and miR-16 (*D*) and luciferase siRNA (*E*). Conditioned medium of HEK293 cells was immunoprecipitated with anti-CD63 antibody or isotype control. The values on the y axis are depicted relative to control, which is arbitrarily defined as 1. *C–E*, each bar is presented as mean S.E. ( $n = 3$ ). n.s. represents not significant.

(ESCRT) machinery because the machinery is highly involved in the exocytosis of some cellular contents and various viruses. On the other hand, Trajkovic *et al.* (18) reported that exosomes are released independently from ESCRT machinery but triggered by sphingolipid ceramide. Thus, it is still unclear whether many types of exosomal contents, such as proteins, lipids, and nucleic acids, are released by the same mechanism. To address the question as to whether miRNA secretion is regulated by the ceramide-dependent pathway or the ESCRT pathway, we first treated HEK293 cells with an nSMase inhibitor, GW4869, which is known to inhibit ceramide biosynthesis (18). As a result of this treatment, extracellular endogenous miR-16 and exogenous miR-146a were dose-dependently reduced, whereas their cellular expression levels remained unchanged (Fig. 3A; supplemental Fig. 3). Consistently, exosomal protein was also decreased by the treatment of GW4869 in a dose-dependent manner (Fig. 3B). Furthermore, a reduction in the signals of an exosome marker, CD63, was observed by immunoblotting of

exosomes purified from the supernatant of the same number of GW4869-treated HEK293 cells when compared with control cells (Fig. 3C, upper panel). Nevertheless, the amounts of CD63 protein were unchanged in exosomes from GW4869-treated cells when the same amounts of exosomal proteins were analyzed (Fig. 3C, lower panel). These data show that the treatment of GW4869 reduces the amount of exosomes released into the conditioned medium but does not modify exosomal protein composition.

We next employed an RNA interference approach to knock down the expression of endogenous nSMase2 in HEK293 cells. A transfection of an siRNA duplex targeting human nSMase2 mRNAs resulted in a robust reduction of nSMase2 protein levels in HEK293 cells (Fig. 4A). Under these conditions, secretion of endogenous miR-16 as well as exogenous miR-146a was attenuated by the nSMase2 siRNA as compared with control cells where negative control siRNAs were transfected, whereas cellular expression of miRNAs was not changed (Fig. 4B). Fur-



**FIGURE 3. Secretion of miRNA was attenuated by GW4869.** *A*, secretion of miRNAs was suppressed by the treatment with GW4869. HEK293 cells were transfected with pri-miR-146a vector in a 6-well plate. The cells were reseeded 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-16 and -146a. The values on the y axis are depicted relative to the amount of each miRNA at 0  $\mu\text{M}$  GW4869, which is arbitrarily defined as 1. *B*, after the treatment with the indicated concentrations of GW4869 for 24 h, the total amounts of proteins in the exosomal pellet purified from large scale cultures of stably miR-146a-transduced HEK293 cells were quantified by a BCA assay and are presented as the values per 10 million secreting cells. *A* and *B*, each bar is presented as mean S.E. ( $n = 3$ ). *C*, purified exosomes secreted by equal numbers of control or GW4869-treated HEK293 cells and equal amounts of total exosomal proteins (quantified by BCA assay) secreted by either control or GW4869-treated HEK293 cells were analyzed by immunoblotting for the presence of CD63 protein. (\*,  $p < 0.05$ , \*\*,  $p < 0.005$ , as compared with untreated cells; Student's *t* test).

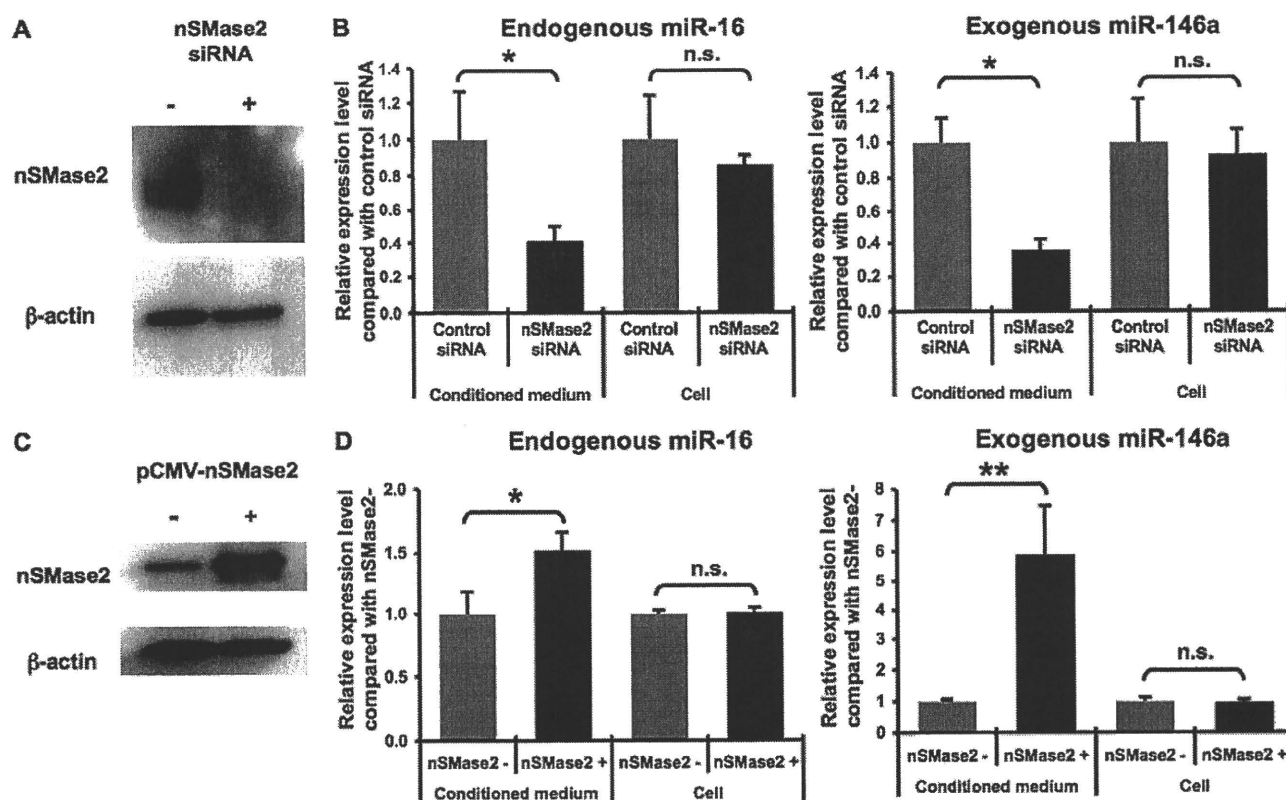
thermore, to complement the results of this knockdown study, we observed that overexpressed human nSMase2 induced the secretion of endogenous miR-16 as well as exogenous miR-146a and luciferase siRNA from HEK293 cells without affecting cellular amounts of the miRNAs (Fig. 4, *C* and *D*, and supplemental Fig. 4A). Although it was already known that nSMase2 overexpression increases the amount of ceramide in the raft fraction and subsequently induces an apoptotic cell death (19), we did not observe any elevated caspase-3/7 activities in nSMase2-overexpressing cells (supplemental Fig. 4B). These results suggest that a ceramide-dependent secretory pathway is involved in miRNA secretion.

**The Secretion of miRNAs Does Not Require ESCRT System**—Gibbins *et al.* (17) showed that a knockdown of some ESCRT-associated proteins, including Alix, vps36, and hrs, deteriorated ESCRT function, resulting in an inhibition of the activity of cellular miRNAs. To examine whether Alix is involved in the secretory process of miRNAs, the knockdown experiment was performed in HEK293 cells carrying a sensor vector. As shown in Fig. 5B, *upper schematic*, our sensor vector is available for assessing miR-146a specific activity, which expresses firefly luciferase and *Renilla* luciferase containing complementary miR-146a sequences at its 3'-untranslated region, allowing us to simultaneously monitor transfection efficiency and miR-146a activity. Consistent with the previous finding, the depletion of Alix by an siRNA transfection fully restored the

miR-146a-suppressed *Renilla* luciferase activity (Fig. 5, *A* and *B*; compare lanes 1, 2, and 4). In other words, ESCRT integrity was compromised by the Alix knockdown. On the contrary, nSMase2 siRNA had no effect on the gene silencing by miR-146a, suggesting that ceramide synthesis is irrelevant to ESCRT function (Fig. 5B; compare lanes 1 and 3). The result in the setting of non-transfection of miR-146a ruled out the possibility that the application of these siRNAs changed the basal *Renilla* luciferase activities (Fig. 5B; compare lanes 4, 5, and 6). QRT-PCR analysis showed that the blocked RNA silencing by the Alix knockdown was not because of the decreased amount of cellular miR-146a (Fig. 5C). Intriguingly, in the HEK293 cells where the ESCRT system was impaired by the Alix siRNA, we did not detect any significant difference of the amount of extracellular miR-146a as compared with a control transfectant (Fig. 5D). Taken together with the results of Figs. 3 and 4, it is unequivocally demonstrated that secretion of miRNAs depends on a ceramide-triggered secretory mechanism but not ESCRT machinery.

**Secretory Small RNAs Can Be Transferred to Recipient Cells**—Our finding that miRNA secretion is tightly regulated by ceramide biosynthesis inspires us to envisage that extracellular miRNAs are not just discards from the originating cells but biologically active molecules. In fact, many reports suggest that small RNAs migrate beyond their cell origin and function in the recipient cells (6, 9). To test whether or not artificial small RNA can be transported into recipient cells under our experimental conditions, we treated firefly luciferase stably expressing HEK293 cells with a conditioned medium enriched in the luciferase siRNAs. The amounts of small RNAs in the conditioned medium were evaluated by QRT-PCR as described under "Experimental Procedures." As a result, the luciferase activities were negatively regulated by the treatment of conditioned medium containing 200 pM luciferase siRNAs as compared with control conditioned medium and fresh RPMI medium as well as miR-146a-enriched conditioned medium (Fig. 6A). In addition to artificial siRNAs, we next examined a transfer of a class of naturally occurring small RNAs, miRNAs. To measure a direct regulation of gene expression by extracellular miRNAs, we performed a reporter assay based on the sensor vector according to an experimental scheme illustrated in Fig. 6B. As shown in Fig. 6C, the normalized *Renilla* luciferase activities were dose-dependently reduced by the treatment of miR-146a-enriched conditioned medium derived from COS-7 and HEK293 cells transfected with pri-miR-146a expression vec-

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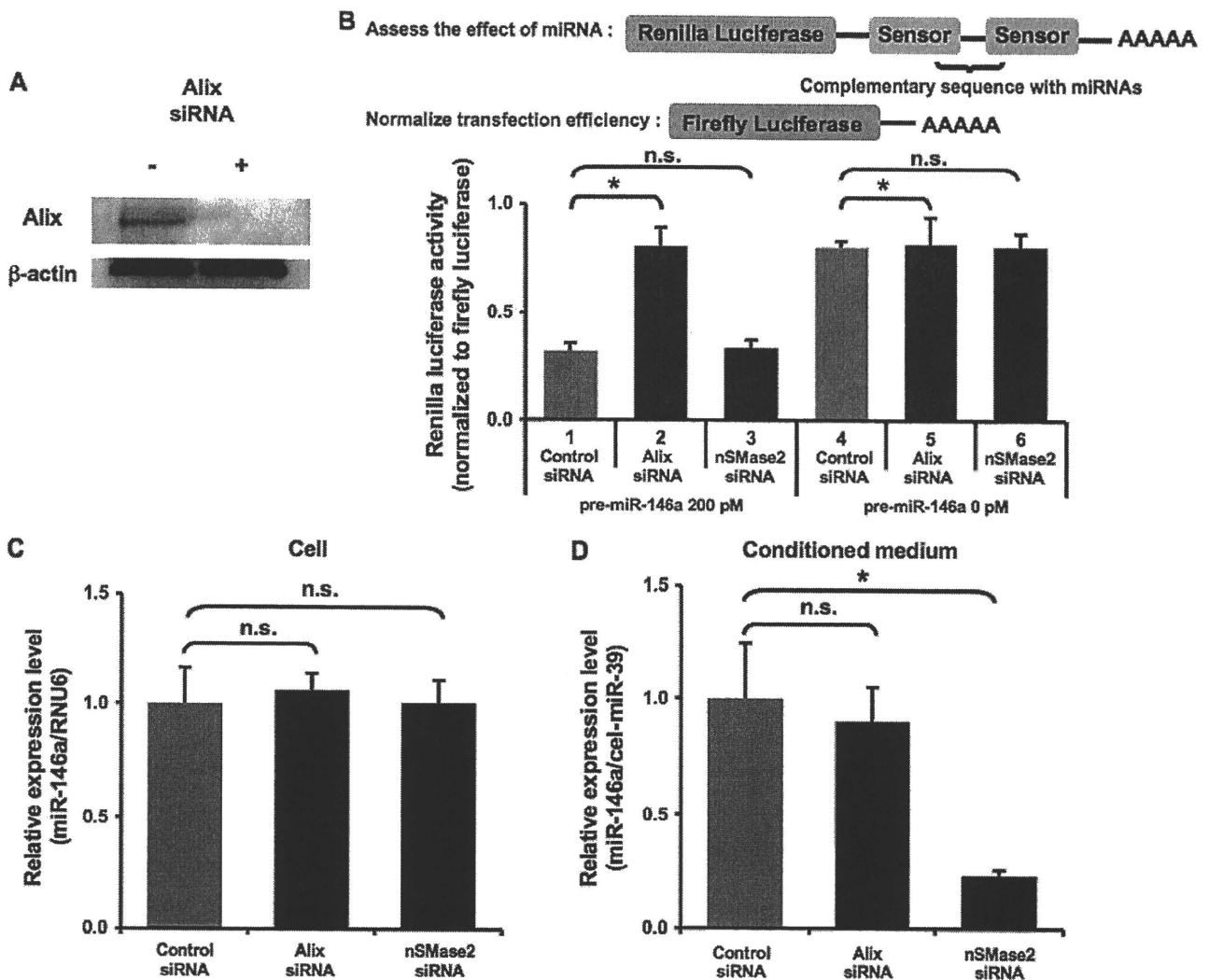


**FIGURE 4. miRNAs were secreted through nSMase2-dependent pathway.** A and B, siRNA-mediated knockdown of nSMase2. HEK293 cells were transfected with pri-miR-146a vector in a 6-well plate. One day later, these cells were reseeded in a 24-well plate and transfected with either negative control or nSMase2 siRNA. The following day, conditioned medium and cell extract were applied to QRT-PCR for miR-16 as well as miR-146a (B). In parallel, cell extract was applied to immunoblot for nSMase2 and  $\beta$ -actin proteins (A). The values on the y axis are depicted relative to the amount of miRNAs of control, which is arbitrarily defined as 1. C and D, augmentation of miRNA secretion by overexpressed human nSMase2. HEK293 cells were transfected with human nSMase2 expression vector or control vector, along with pri-miR-146a vector. After a 24-h incubation, conditioned medium and cell extract were applied to QRT-PCR (D), and cell extract was applied to immunoblot (C). The values on the y axis are depicted relative to the amount of miRNAs of nSMase2<sup>-</sup>, which is arbitrarily defined as 1. B and D, each bar is presented as mean S.E. ( $n = 3$ ). (\*,  $p < 0.05$ , \*\*,  $p < 0.005$ , as compared with control cells; Student's  $t$  test); n.s. represents not significant.

tors. This effect was not due to a transfer of free miRNAs remaining in cellular debris and conditioned medium because a direct addition of 100 nM naked miR-146a analogues did not change the luciferase activity (supplemental Fig. 5). Furthermore, to exclude the possibility that the inhibitory effect is independent of the specific interaction between the transferred miR-146a and the complementary binding sites in the sensor vector, we used a mutated miR-146a sensor vector constructed as described under "Experimental Procedures." As shown in Fig. 6D, the normalized *Renilla* luciferase activities from the mutated sensor vector were not changed by the addition of the conditioned medium from miR-146a-overexpressing HEK293 cells. These results show that extracellular miRNAs packaged in secretory exosome vesicles can be delivered into recipient cells and act as physiologically functional molecules to exert gene silencing through the same mechanism as cellular miRNAs.

**Secretory Tumor-suppressive miRNAs Attenuate PC-3M Cell Proliferation**—To gain further insight into the biological activity of secretory miRNAs, we investigated whether they can make an impact on a cellular phenotype. miR-146a is known to be down-regulated in prostate cancer (20), and reconstitution of its expression results in growth inhibition of prostate cancer cells (21). As schematically depicted in Fig. 7A, supernatants

containing transduced miR-146a derived from COS-7 cells were added to the culture medium of metastatic prostate cancer cell line PC-3M-luc cells. This cell line stably harbors a firefly luciferase gene, enabling us to assess cell numbers with the luciferase activity. After a 3-day incubation, PC-3M-luc cells showed an  $\sim 20\%$  decrease in proliferation in the presence of 21 pM miR-146a in the conditioned medium, whereas liposomally transfected synthetic miRNAs at 10 pM suppressed cell growth to a similar extent (Fig. 7B). To determine whether extracellular miRNA exerts regulatory action on its target gene, we examined the expression of ROCK1, a target gene for miR-146a, in the treatment with extracellular miR-146a (21). The addition of the conditioned medium of miR-146a-transduced COS-7 cells significantly knocked down ROCK1 protein expression in PC-3M-luc cells to the same degree as the miR-146a analogue molecule under conditions where the amount of  $\beta$ -actin was not affected (Fig. 7C). To confirm that the cell growth inhibition resulted from the transferred exosomal miR-146a, we examined whether the suppressive effect can be cancelled by a reduced exosome secretion. Conditioned medium from GW4869-treated donor cells had no inhibitory activity on cell proliferation after a 4-day incubation (Fig. 7D; compare lanes 1 and 2). We also did not observe any direct effect of GW4869 on the restoration of miR-146a-suppressed cell



**FIGURE 5. The secretion of miRNAs does not require ESCRT system.** *A*, knockdown of human Alix protein. HEK293 cells transfected with negative control siRNA or Alix siRNA were harvested and applied to immunoblot for Alix and  $\beta$ -actin proteins. *B*, the depletion of Alix impaired miRNA activity. *Upper schematic*, our sensor vector possesses *Renilla* luciferase and firefly luciferase for assessing the miRNA activity and for normalizing transfection efficiency, respectively. *Lower graph*, HEK293 cells were transfected with 0.1  $\mu$ g of miR-146a sensor vector and the indicated siRNAs together with 200 or 0 pM pre-miR-146a in a 96-well plate. The following day, the cells were applied to a Dual luciferase reporter assay. The values on the y axis are normalized *Renilla* luciferase activity. *C* and *D*, cellular and extracellular miR-146a were not affected by Alix siRNA. MiR-146a stably overexpressing HEK293 cells were transfected with the indicated siRNAs. After the medium switch, the cells were cultured for another 24 h, and the cell lysate (*C*) and conditioned medium (*D*) were then applied to miRNA QRT-PCR analysis. The values on the y axis are depicted relative to control siRNA, which is arbitrarily defined as 1. *B–D*, each bar is presented as mean S.E. ( $n = 3$ ). (\*,  $p < 0.05$ ; \*\*,  $p < 0.005$ , as compared with control siRNAs; Student's *t* test); n.s. represents not significant.

growth (Fig. 7D; compare lanes 3 and 4). These findings suggest that itinerant exosomal miRNAs can induce some phenotypic changes in the recipient cells.

## DISCUSSION

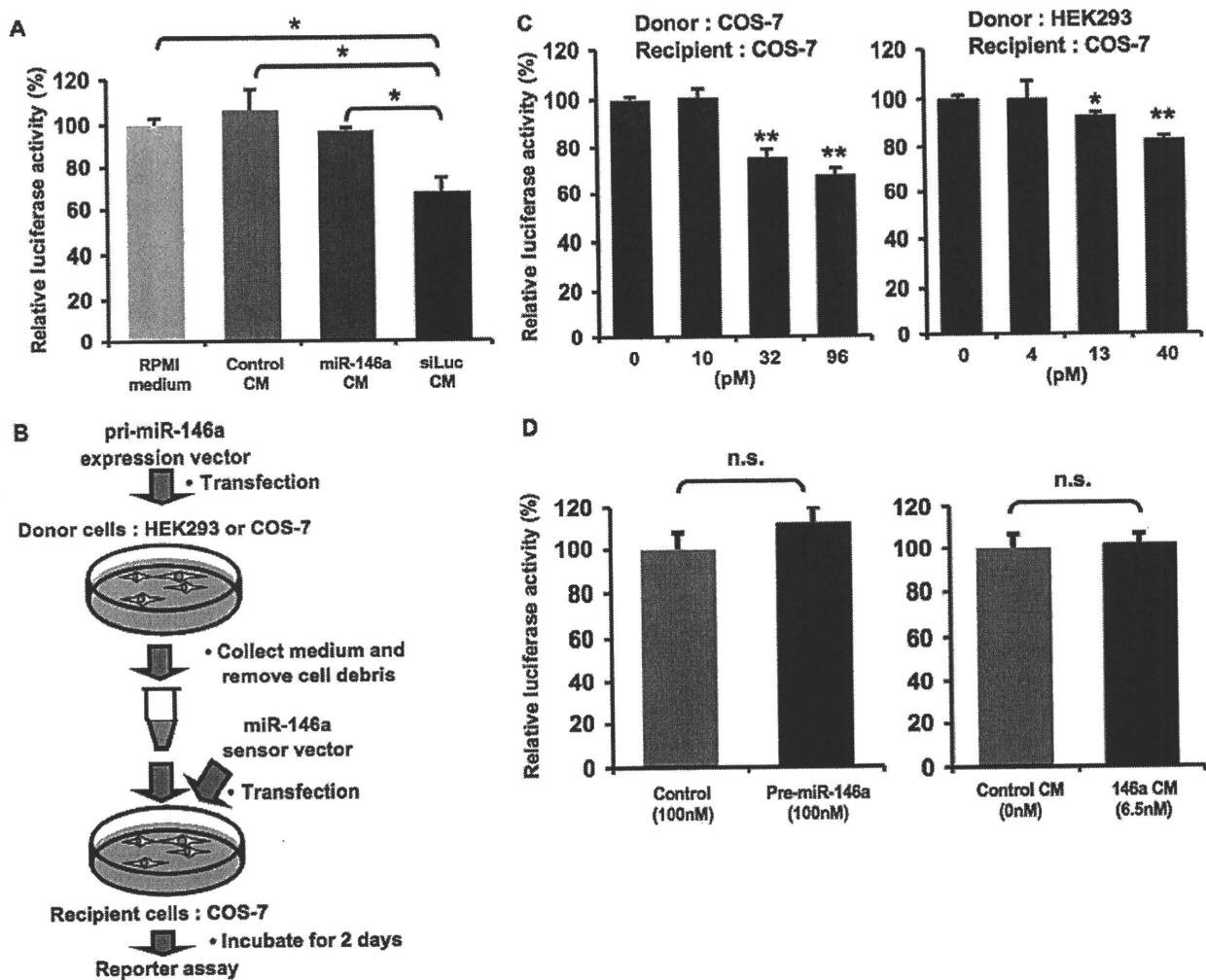
Our data provide a fresh insight into the secretory machinery of miRNAs. Through our mechanistic studies with a chemical inhibitor as well as nSMase2 overexpression and knockdown, we contend that miRNAs can be incorporated into exosomes and released via a ceramide-dependent pathway independently of ESCRT machinery. It remains elusive, however, how miRNAs are sorted into exosomes for their secretion. Two recent studies provide evidences that ESCRT complex associates with components of miRNA effector complexes and that RNA silencing takes place on MVB (22).

Taken together, MVB could be a crossroads of miRNAs bound for the secretory pathway and gene-silencing cycle. Further investigations are needed to unveil a sorting mechanism by which miRNAs are determined to be secreted or to stay and function in their originating cells.

There is growing evidence that secretory miRNAs play a role in a variety of physiological phenomena. Zernecke *et al.* (9) showed that the transfer of miR-126 loaded into endothelial apoptotic bodies induced the expression of CXCL12 and the recruitment of progenitor cells, thereby alleviating atherosclerosis. Additionally, Rechavi *et al.* (8) proposed that viruses could tamper with the host immune response by transportation of viral non-autonomously encoded small RNAs. Both vascular protection and manipulation of the immune system by viruses occur for a long term. These find-



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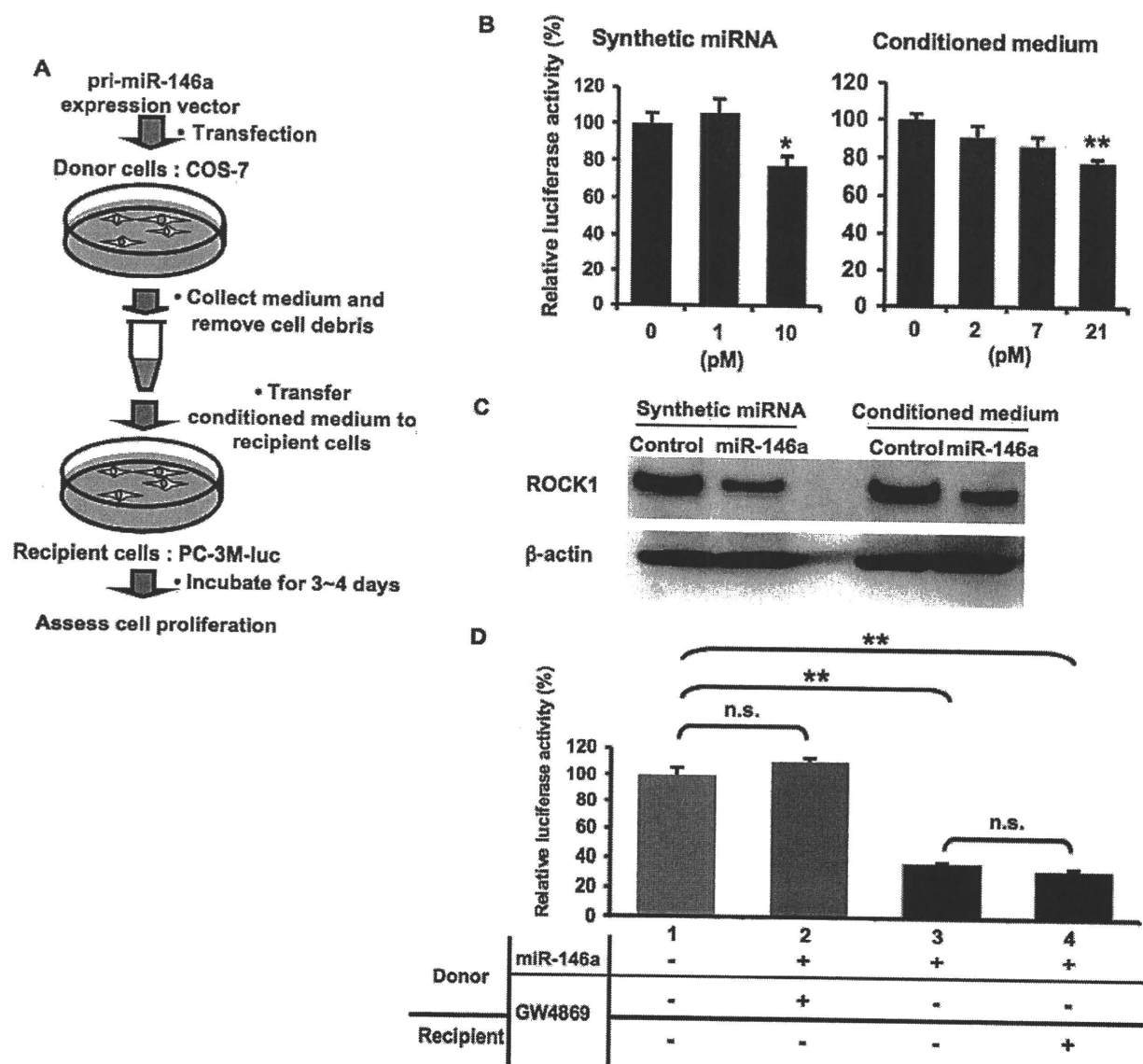


**FIGURE 6. Secretory small RNAs can be transferred to recipient cells.** *A*, treatment with conditioned medium enriched in luciferase siRNA down-regulated luciferase activity. HEK293 cells stably transfected with firefly luciferase were incubated with the indicated medium for 3 days. The conditioned medium was prepared from HEK293 cells transfected with psiRNA-LucGL3 or empty vector. The luciferase siRNA was not detected in RPMI medium and control CM as well as miR-146a CM using QRT-PCR, whereas luciferase siRNA (*siLuc*) CM contained 200 pM luciferase siRNA as calculated under "Experimental Procedures." The concentration of miR-146a in miR-146a CM was 123 pM. The cells were harvested and applied to luciferase reporter assay. The values on the y axis are depicted relative to firefly luciferase activity of RPMI medium-treated cells, which is defined as 100%. *B*, the diagram shows a reporter assay for testing the direct regulation of target gene expression by extracellular miRNAs. *C*, extracellular miR-146a derived from COS-7 and HEK293 cells suppressed luciferase activity of the sensor vector. COS-7 cells transfected with miR-146a sensor vector were used as recipient cells. The recipient cells were incubated in the conditioned medium containing extracellular miRNAs of the indicated concentrations. After a 2-day incubation, luciferase reporter assay was performed as described under "Experimental Procedures." *D*, miR-146a did not reduce luciferase activity from the mutated sensor vector. COS-7 cells transfected with the mutated miR-146a sensor vector were used as recipient cells. The recipient cells were transfected with synthetic miRNAs (*left graph*) or incubated in the conditioned medium containing extracellular miRNAs at the indicated concentrations (*right graph*). Luciferase assay was carried out as described above. *C* and *D*, the values on the y axis are depicted relative to normalized *Renilla* luciferase activity of control cells, which is defined as 100%. *A*, *C*, and *D*, each bar is presented as mean S.E. ( $n = 3$ ). (\*,  $p < 0.05$ , \*\* $p < 0.005$ , as compared with control recipient cells; Student's *t* test); *n.s.* represents not significant.

ings prompt us to conceive of the idea that secretory miRNAs could mainly serve in chronic biological events, such as the formation of a tumor microenvironment.

Many tumors have a remarkable ability to mold their stromal environment to their own advantage (23, 24). Recent studies show the importance of communication between cancer cells and their surroundings through shedding of membrane exosomes, which can fuse to cells in the vicinity (7, 25, 26). For instance, epidermal growth factor receptor VIII proteins were transferred into glioma cells lacking epidermal growth factor receptor VIII via secretory membrane microvesicles (26). Together, membrane microvesicles or exosomes of cancer cells can contribute to a horizontal prop-

agation of oncogenes and their associated transforming phenotype among subsets of cancer cells. Here, we show that re-expression of miR-146a through intercellular transfer leads to cell growth inhibition of PC-3M cells, where the expression of the miRNA is at a very low level. It is well known that many kinds of miRNAs are down-regulated in cancer cells, resulting in tumorigenesis, tumor progression, and metastasis (27). We therefore hypothesize that down-regulated miRNAs in cancer cells are compensated during the initial stage of tumorigenesis by the surrounding cells that supply exosomes containing the decreased miRNAs. However, once the surrounding cells cannot meet the demand, cancer cells end up entering an advanced stage.



**FIGURE 7. Secretory tumor-suppressive miRNAs attenuated PC-3M cell proliferation.** *A*, schematic representation of a cell proliferation assay. *B*, cell growth inhibition by synthetic miR-146a and extracellular miR-146a. PC-3M-luc cells were transfected with synthetic miRNAs (*left graph*) or incubated in the conditioned medium containing extracellular miRNAs at the indicated concentrations (*right graph*) followed by cell growth assay as described under "Experimental Procedures." *C*, miR-146a-mediated ROCK1 suppression in PC-3M-luc cells. The final concentrations of synthetic and extracellular miR-146a are 10 and 21 pM, respectively. *D*, the treatment with GW4869 to donor cells restored the reduced cell growth by the exosomal miR-146a. Donor COS-7 cells transfected with pri-miR-146a expression vector were incubated in the presence (*lane 2*) or absence of 10  $\mu$ M GW4869 for 3 days. The conditioned medium from COS-7 cells transfected with empty vector was used as a control (*lane 1*). GW4869-untreated conditioned medium enriched in miR-146a was divided into two aliquots, one of which was treated with 10  $\mu$ M GW4869 (*lane 3*) and the other of which was not treated (*lane 4*) before the transfer to recipient cells. The following assay was conducted as described above. *B* and *D*, the values on the y axis are depicted relative to normalized *Renilla* luciferase activity of control cells, which is defined as 100%. Each bar is presented as mean S.E. ( $n = 3$ ). (\*,  $p < 0.05$ ; \*\*,  $p < 0.005$ , as compared with untreated PC-3M-luc cells; Student's *t* test); *n.s.* represents not significant.

Secretory miRNAs could be conducive to the maintenance and surveillance system against cancer progression.

Small RNAs including antisense, siRNA, and miRNA are emerging as promising therapeutic agents against a wide array of diseases (28). Effective delivery of these molecules is crucial to their successful clinical application. Now, exosomes have entered the limelight in delivery of therapeutic nucleic acids with their ability to transfer exogenous miRNAs into tumor cells. Exosomes are naturally produced membrane vesicles that circulate in the bloodstream and are

taken up by various types of cells. These aspects might be beneficial for the improvement of durability, stability, and safety for *in vivo* delivery. In fact, tumor exosomes generated tumor regression *in vivo*, acting as a cancer vaccine through their capacity of antigen presentation (29). It is essential to examine whether exosomes can deliver their cargoes in an *in vivo* animal model.

In conclusion, we revealed the secretory machinery of miRNAs and their intercellular transfer. Our results raise the possibility that in the animal kingdom, circulating miRNAs

## Regulation of MicroRNA Secretion

could play a pivotal and general role as a signaling molecule in physiological and pathological events. We anticipate that our study could pave the way for explorations of signal network mediated by secretory miRNAs.

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SHORT REPORT

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# microRNA as a new immune-regulatory agent in breast milk

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## Abstract

**Background:** Breast milk is a complex liquid that provides nutrition to the infant and facilitates the maturation of the infant's immune system. Recent studies indicated that microRNA (miRNA) exists in human body fluid. Because miRNAs are known to regulate various immune systems, we hypothesized that human breast milk contains miRNAs that may be important for the development of the infant's immune system.

**Findings:** We profiled miRNA expression in human breast milk and detected high expression levels of immune-related miRNAs in the first 6 months of lactation. Furthermore, these miRNA molecules are stable even in very acidic conditions, indicating that breast milk allows dietary intake of miRNAs by infants.

**Conclusions:** Our findings provide new insight into how breast milk can modulate the development of the infant's immune system. This study suggests the transfer of genetic material as miRNA from human to human occurs by means other than through sexual reproduction.

## Introduction

The mammary glands of mammals are specialized organs whose function is to produce milk, the primary source of nutrition for newborns. Breastfeeding is recognized as one of the most valuable contributors to infant health [1]. Human breast milk protects infants not only against infections but also against chronic diseases. Furthermore, human breast milk contains certain growth factors that help the infant intestine to develop, become able to absorb milk and prepare for food intake. When maternal breast milk is unavailable, the alternative is infant formula. Compared with infants fed on formula, infants fed on breast milk have a lower incidence of digestive problems and are more likely to be protected against gastrointestinal and respiratory infections. Despite the fact that breastfeeding is known to be the best method for nourishing infants, how exactly breastfeeding works to provide the best nutrition and protect infants against disease is not fully understood.

Many immune-related substances are present in human breast milk, and their effects upon the recipient infants are widely recognized [2,3]. For instance, human breast milk contains large quantities of secretory (s)IgA.

These antibodies can bind to pathogens and prevent their attachment to an infant's cells. Furthermore, human breast milk contains measurable levels of leukocytes. In addition to these immunologic components, breast milk contains several nonspecific factors, such as lysozyme, lactoferrin and oligosaccharides, which have antimicrobial effects. Lysozyme inhibits the growth of many bacterial species by disrupting the proteoglycan layer of the bacterial cell wall. Lactoferrin, known as a multifunctional protein in human breast milk, also limits bacterial growth by removing essential iron and by stimulating cytokine production, and enhancing mucosal immunity, natural killer (NK) cell activity and macrophage cytotoxicity. Substantial amounts of oligosaccharides in the mammary gland were found in human breast milk, and these block attachment of microbes to an infant's mucosa, preventing infections. Nucleotides in human breast milk have been shown to enhance the immune function in infants [4]. However, several additional immune regulatory components in milk may explain why breastfeeding can reduce infant mortality.

MicroRNAs (miRNAs) are small regulatory RNA molecules that modulate the activity of specific mRNA targets and play important roles in a wide range of physiologic and pathologic processes [5-7]. Specific miRNAs that play important roles in a wide range of physiologic

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and pathologic processes in mammals may be involved in the control of immunologic reactions [6,7]. A loss-of-function approach indicated that miRNAs are crucially involved in the *in vivo* control of immune regulation including cellular differentiation and immune response [8-10]. Recently, miRNAs have been found in serum, plasma and other body fluids [11-13]. Serum miRNAs can serve as potential biomarkers for the detection of various cancers and other diseases. Furthermore, it was reported that miRNAs circulate in plasma microvesicles in peripheral blood in healthy people [14]. The majority of the plasma microvesicles from normal individuals are derived from blood cells. However, the physiologic roles of body fluid miRNAs are undetermined. In this report, we show that a considerable number of miRNAs, especially those that function in the immune system, are found in human breast milk. Furthermore, we detected a higher expression of immune-related miRNAs in the first 6 months of lactation. These miRNAs are stable even under harsh conditions. Our findings suggest that human breast milk contains miRNAs capable of transfer to immune cells to support the development of an infant's immune system.

## Results

### Extraction of RNAs and expression analysis

To verify the existence of miRNAs in human breast milk, we extracted total RNA from human breast milk. miRNAs were detected in each individual at concentrations ranging from 9.7 ng/ml to 228.2 ng/ml. The samples contained a substantial amount of RNA, but no or only very low amounts of ribosomal RNA (18S and 28S) (Figure 1a). Furthermore, large amounts of small RNA (<300 nucleotides) were detected in the milk.

Next we performed a miRNA microarray to show the existence of miRNAs in the milk. Using microarray analysis, 281 of 723 known human miRNAs were detected. Interestingly, several immune-related miRNAs were abundant in the milk (Figure 1b): miR-155, a regulator of T- and B-cell maturation and the innate immune response; miR-181a and miR-181b, regulators of B-cell differentiation and CD4+ T-cell selection; miR-17 and miR-92 cluster: a ubiquitous regulator of B-cell, T-cell and monocyte development, miR-125b, a negative regulator of tumor necrosis factor- $\alpha$  production, activation and sensitivity; miR-146b, a negative regulator of the innate immune response; miR-223, a regulator of neutrophil proliferation and activation; and let-7i, a regulator of Toll-like receptor 4 expression in human cholangiocytes. By contrast, T- and B-cell regulatory miR-150 was not detected, and several tissue-specific miRNAs, such as miR-122 (liver), miR-216, miR-217 (pancreas) and miR-142-5p and miR-142-3p (hematopoietic cells), were barely

detectable (Figure 1b). Furthermore, the expression pattern of miRNA within different breast milk samples from the same mother did not differ greatly with time after birth (Figure 1c, d). Between individuals, however, there was more variation. Notably, we detected higher expression of several immune system-related miRNAs from human breast milk in the first 6 months, which is the period before infants receive complementary food (Figure 2).

### Resistance and stability of miRNAs

The existence of RNase in body fluids is already known [13], and thus there should be no intact RNA present. The presence of miRNAs suggests that these miRNA species are resistant to RNase digestion. To prove this possibility, human breast milk was treated with RNase A/T.

Strikingly, treatment with RNase had hardly any effect on human breast milk miRNAs (Figure 3a). By contrast, the profile of total RNA from that milk and exogenously added synthetic miRNAs were degraded by the same treatment (data not shown). These data clearly demonstrate that human breast milk miRNAs are resistant to RNase digestion.

Their stability was further studied after treatment under harsh conditions including freeze-thaw cycles and low pH. The samples were analyzed by quantitative reverse transcription (qRT)-PCR analysis.

Results of qRT-PCR analysis of miRNAs in human breast milk samples subjected to harsh conditions were not significantly different from samples not subjected to these conditions. As shown in Figure 3b, human breast milk miRNAs were resistant to several freeze-thaw cycles. Moreover, human breast milk miRNAs were stable when the milk was treated for 1 h in an acidic (pH 1) solution (Figure 3c).

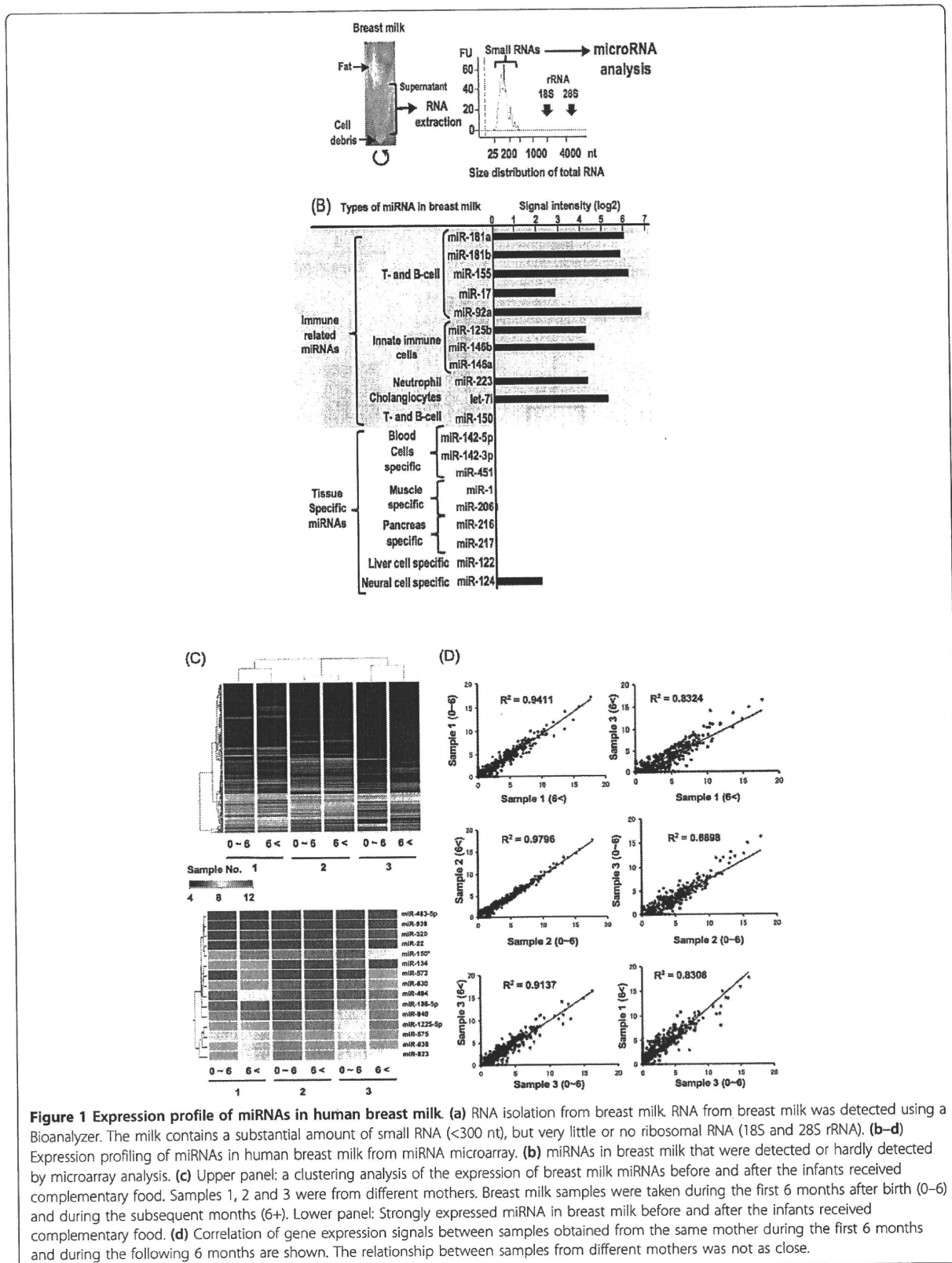
### Existence of microvesicles

Observation of the stability of human breast milk miRNAs *in vitro* suggests the possibility that they are contained within microvesicles or exosomes [14]. To verify this, we isolated the CD63-positive exosome fraction and investigated miRNA expression.

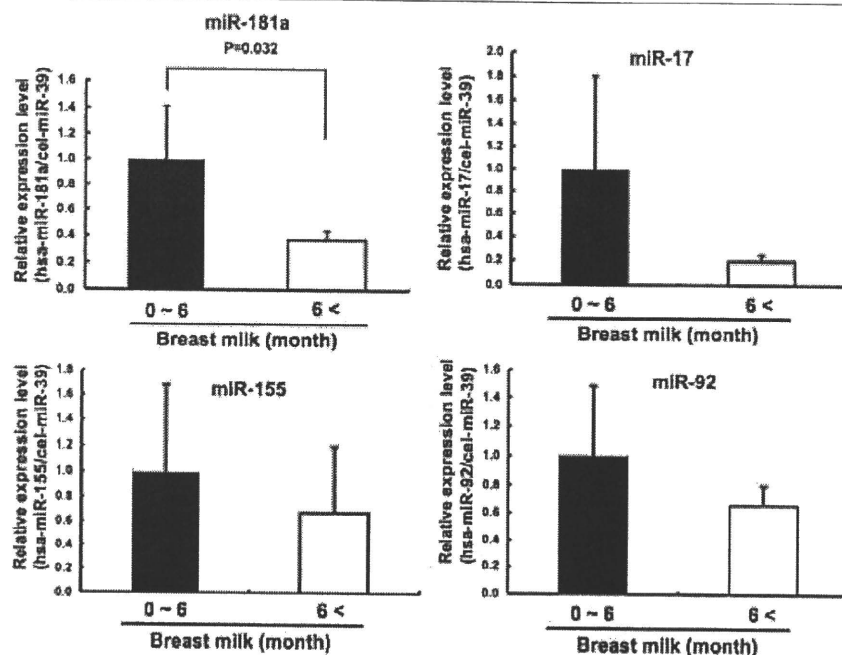
As shown in Figure 3d, miR-181a and miR-17, which we detected by microarray analysis, were also detected in the CD63-positive exosome fraction. However, it is still possible that other miRNA protection mechanisms, such as an apoptotic body, may protect miRNA from harsh conditions.

### Presence of miRNAs in other body fluids

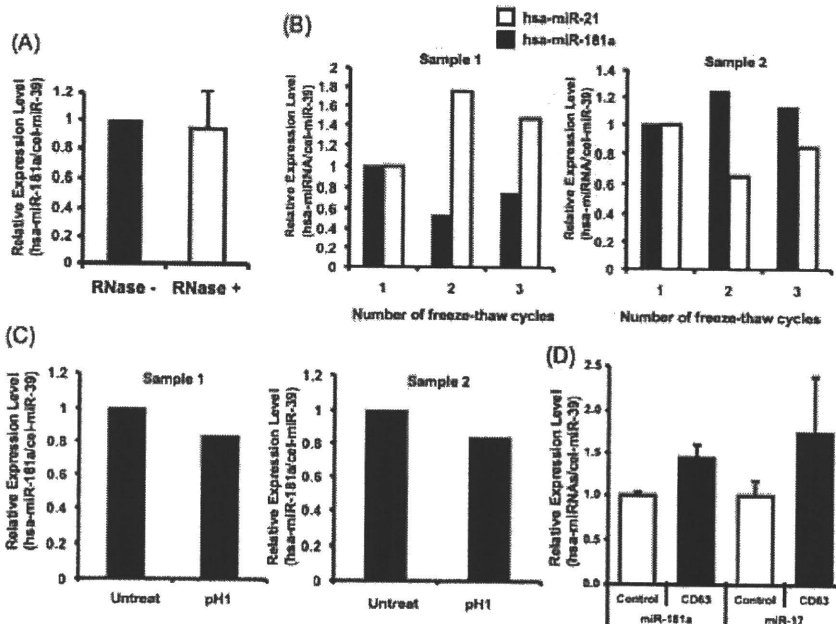
It was previously reported that miRNAs are also detected in serum samples [11-13]. To investigate differences in miRNA expression in different body fluids, we



**Figure 1** Expression profile of miRNAs in human breast milk. (a) RNA isolation from breast milk was detected using a Bioanalyzer. The milk contains a substantial amount of small RNA (<300 nt), but very little or no ribosomal RNA (18S and 28S rRNA). (b-d) Expression profiling of miRNAs in human breast milk from miRNA microarray. (b) miRNAs in breast milk that were detected or hardly detected by microarray analysis. (c) Upper panel: a clustering analysis of the expression of breast milk miRNAs before and after the infants received complementary food. Samples 1, 2 and 3 were from different mothers. Breast milk samples were taken during the first 6 months after birth (0-6) and during the subsequent months (6+). Lower panel: Strongly expressed miRNA in breast milk before and after the infants received complementary food. (d) Correlation of gene expression signals between samples obtained from the same mother during the first 6 months and during the following 6 months are shown. The relationship between samples from different mothers was not as close.



**Figure 2** Comparison by qRT-PCR of immune-related miRNAs in the breast milk obtained before 6 months of age (n = 5) (before the infants received complementary food) and from 6 to 12 months of age (n = 13) (when infants were receiving complementary food).



**Figure 3** General characterization of miRNAs in breast milk. (a-c) The stability of breast milk miRNAs. Total RNA was extracted and then analyzed by qRT-PCR. Breast milk was (a) incubated with RNase A/T for 3 h at 37°C, (b) subjected to three freeze-thaw cycles or (c) treated for 3 h in a low pH solution (pH 1) before RNA extraction. Representative data are shown. (d) Expression of miR-181a and miR-17 derived from CD63-positive exosomes isolated from human breast milk (0.3 ml) from different mothers (n = 4). Human breast milk was immunoprecipitated with anti-CD63 antibody or isotype control.

analyzed expression of several immune-regulated miRNAs in human serum and human breast milk.

Interestingly, the granulocyte-regulated miRNA, miR-223, is the most strongly expressed miRNA in normal human plasma, but its expression in breast milk is low compared with that in serum (Figure 4) [14]. On the other hand, breast milk is rich in miR-181 and miR-155, and these were detected at similar levels in serum. Furthermore, miRNA microarray analysis revealed that expression of miR-146a, which is high in plasma, was not found in breast milk, whereas miR-146b, which is not abundant in plasma was abundantly expressed in breast milk (Figure 1b).

### Discussion

There are several reports in the literature showing the presence of miRNAs in body fluids including blood plasma/serum, saliva and urine [11-13]. These studies showed the usefulness of miRNAs as a biomarker for disease, but did not investigate any biologic function of the miRNAs in body fluid. Furthermore, because the function of breast milk is to provide nutrition to infants from mothers, miRNA could also have functions in an infant's body.

In our study, miRNAs in breast milk were stable even under very acidic conditions (pH 1). This suggests that these molecules can tolerate an infant's gastrointestinal environment and be absorbed in to the intestine, thus influencing the immune system, as the intestine is one of their major immune organs (Figure 3c). The storage and freeze-thawing of breast milk did not denature the miRNAs, a dietetically important finding for low-birth-weight babies and other hospitalized infants who are usually given freezer-stored breast milk (Figure 3b). The resistance of the miRNAs to the RNase treatment indicated that the miRNAs in human breast milk might be packaged inside complexes such as exosomes or microvesicles (Figure 3a) [14,15]. Furthermore, as shown in Figure 3d, we detected miR-181a and miR-17 in a CD63-positive fraction from human breast milk. A previous report suggested that breast milk-derived exosomes can increase the number of Foxp3+ CD4+ CD25+ regulatory T cells in infants [16]. This is consistent with our results, showing that human breast milk is rich in T-cell-regulating miRNAs [17,18]. Furthermore, human breast milk miRNAs may induce B-cell differentiation, because the milk is rich in miR-181 and miR-155, both

