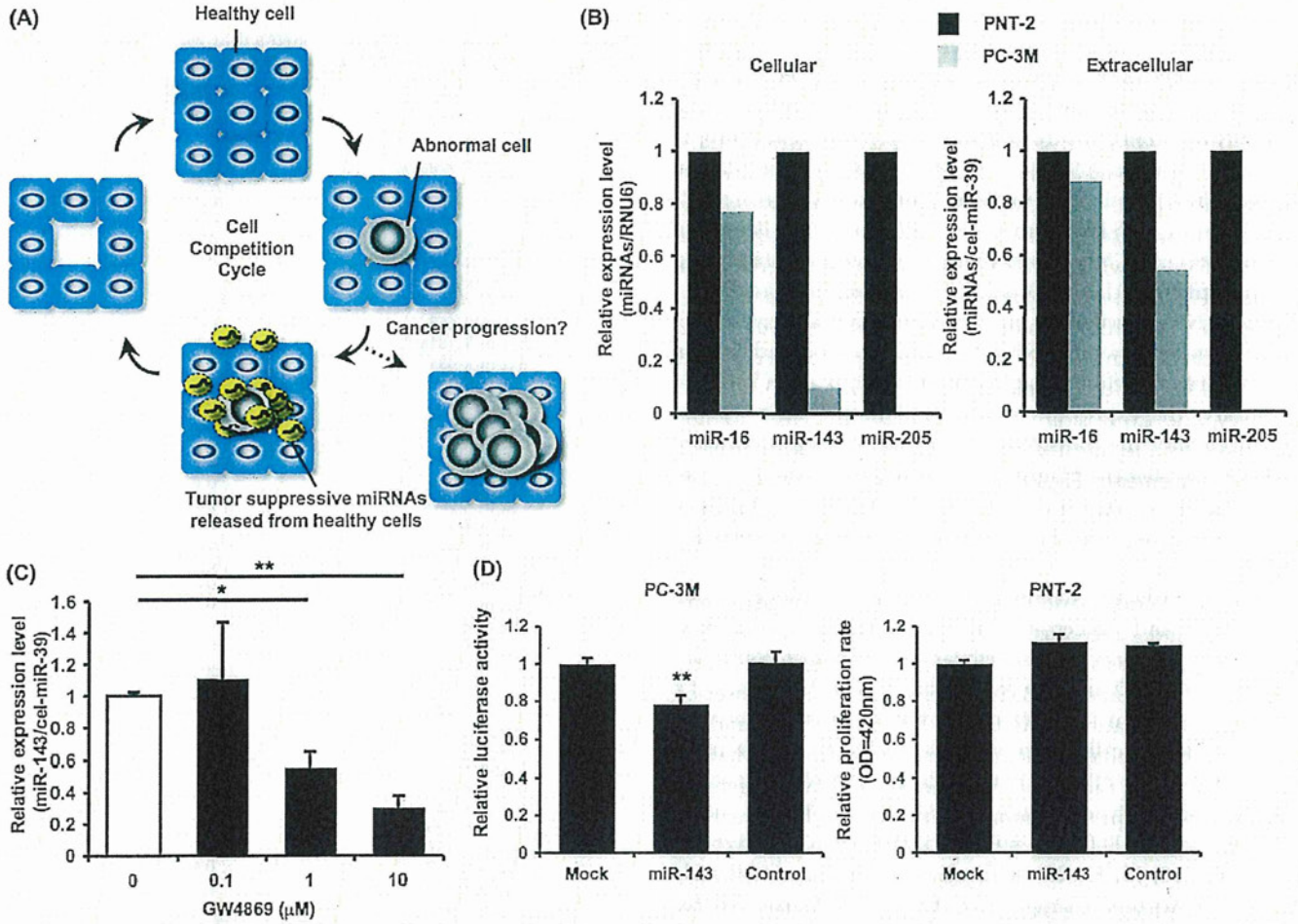

FIGURE 2. **Down-regulation of cellular and extracellular tumor-suppressive miRNAs in PC-3M-luc cells.** *A*, shown is a schematic representation of hypothetical tumor initiation process. Neighboring healthy cells (*blue*) secrete tumor-suppressive miRNAs (*light yellow*) to inhibit the proliferation 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.

Secretory miR-143 as an Anti-cancer Signal

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

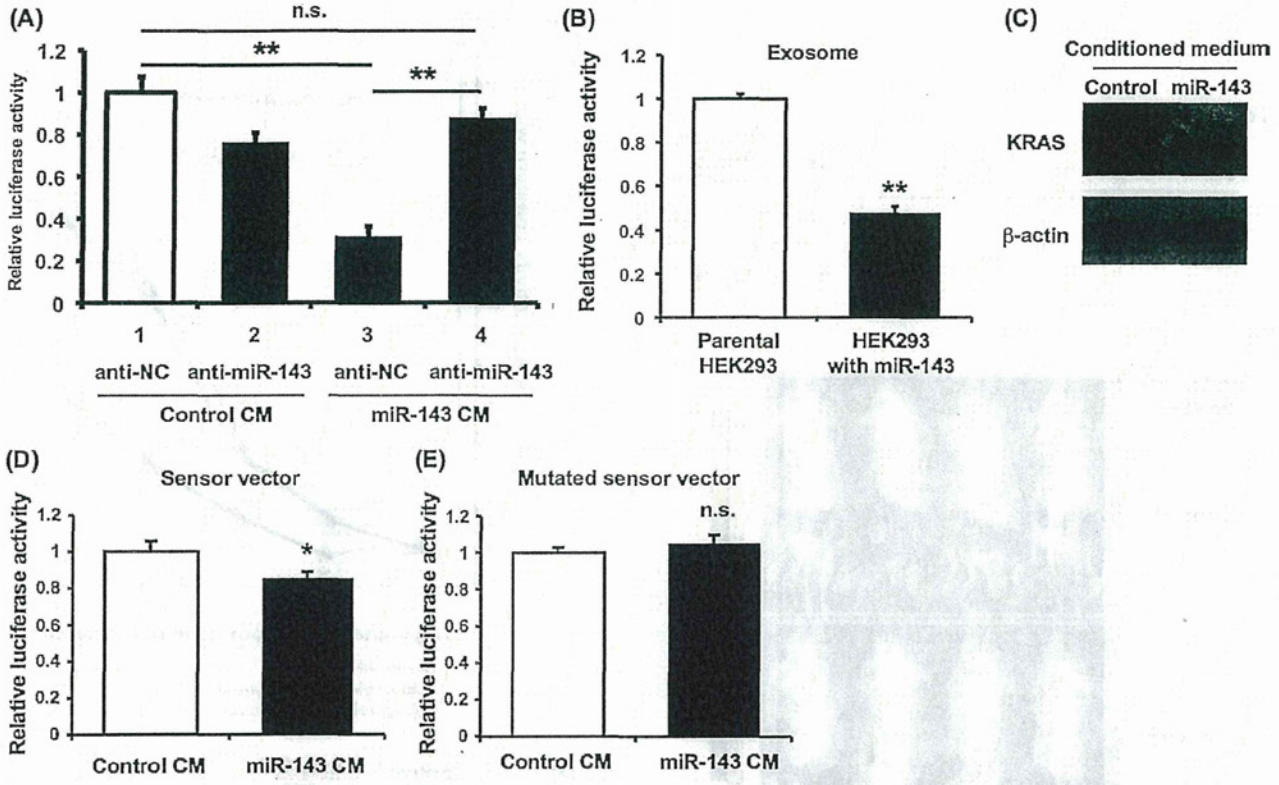

FIGURE 3. **Transfer of secretory miR-143 to PC-3M-luc cells *in vitro*.** *A*, the transfection of anti-miR-143 to PC-3M-luc cells restored the reduced cell growth by the CM derived from miR-143 overproducing cells. After the transfection with 3 nM miR-143 inhibitor molecule (anti-miR-143) (*lanes 2* 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

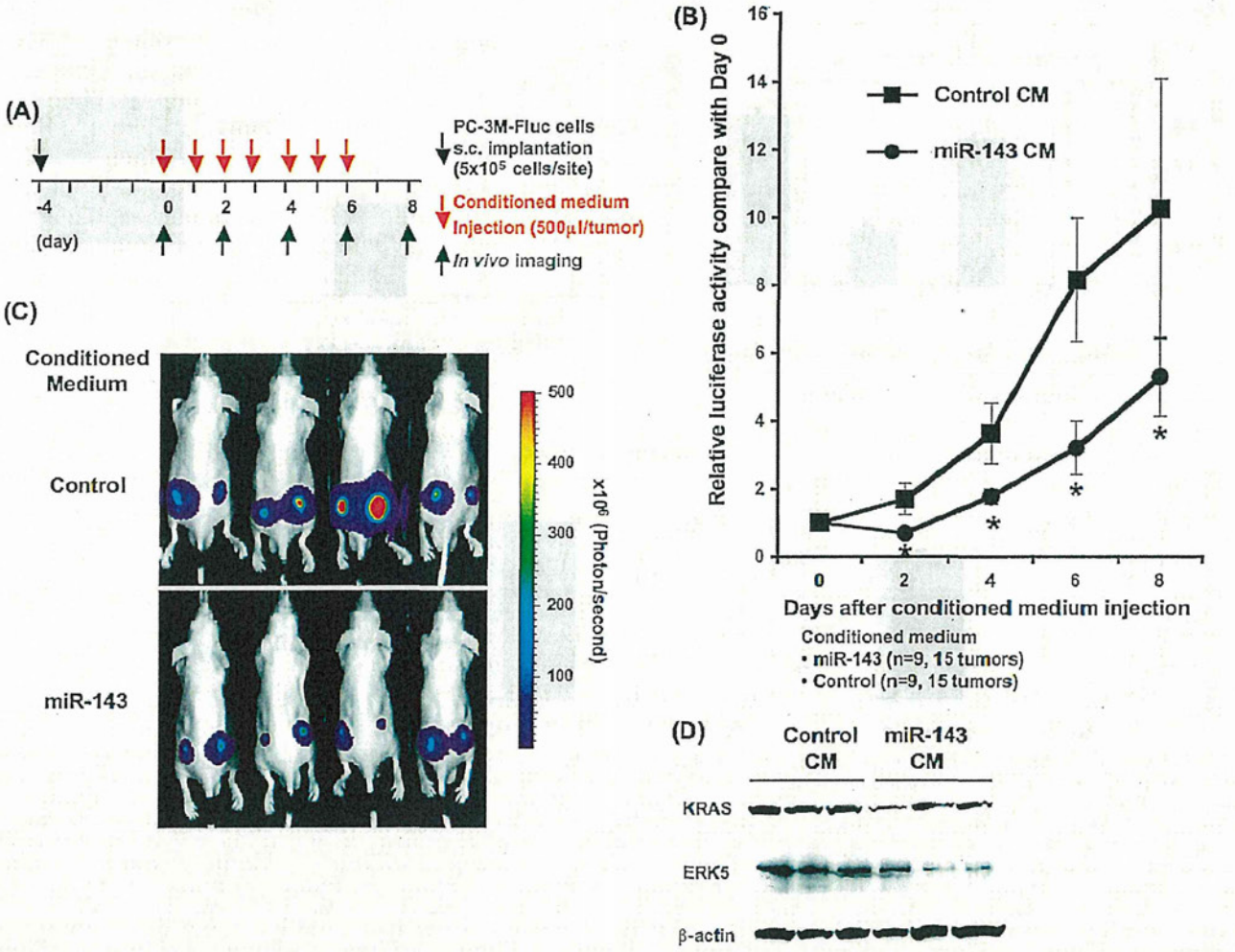

FIGURE 4. **Transfer of secretory miR-143 to PC-3M-luc cells *in vivo*.** *A*, shown is the timetable for conditioned medium injections and *in vivo* imaging. *B*, shown are tumor growth ratios of the inoculated PC-3M-luc cells during the secretory miR-143 treatment. *Closed circles* and *closed squares* 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

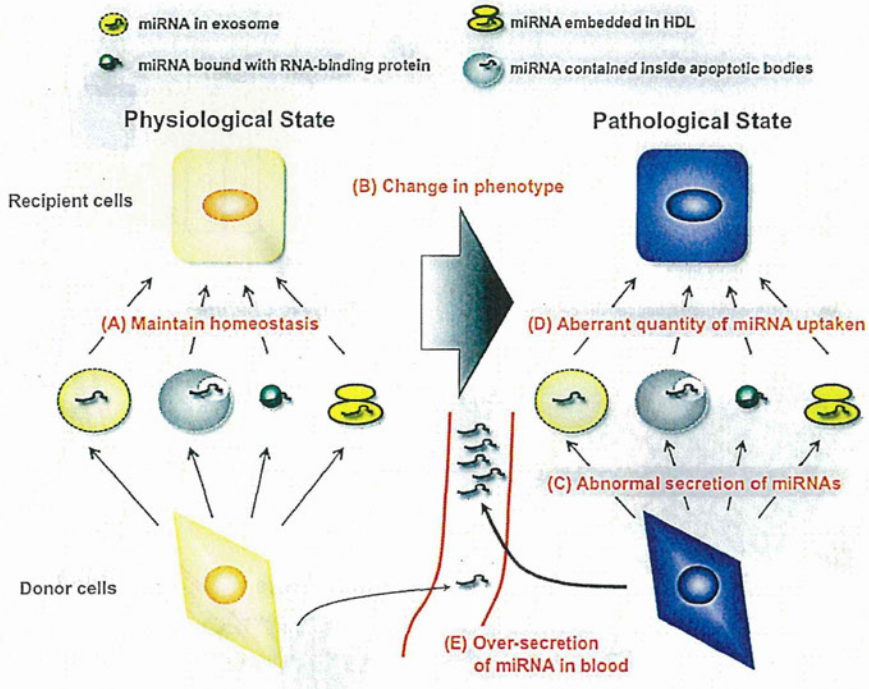

FIGURE 1 | Unveiling the mechanisms and roles of secretory miRNAs in both physiological and pathological conditions is essential for the development of novel diagnostic methods and therapeutics to treat various human diseases. In the physiological condition, secretory miRNAs may maintain the homeostasis 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.

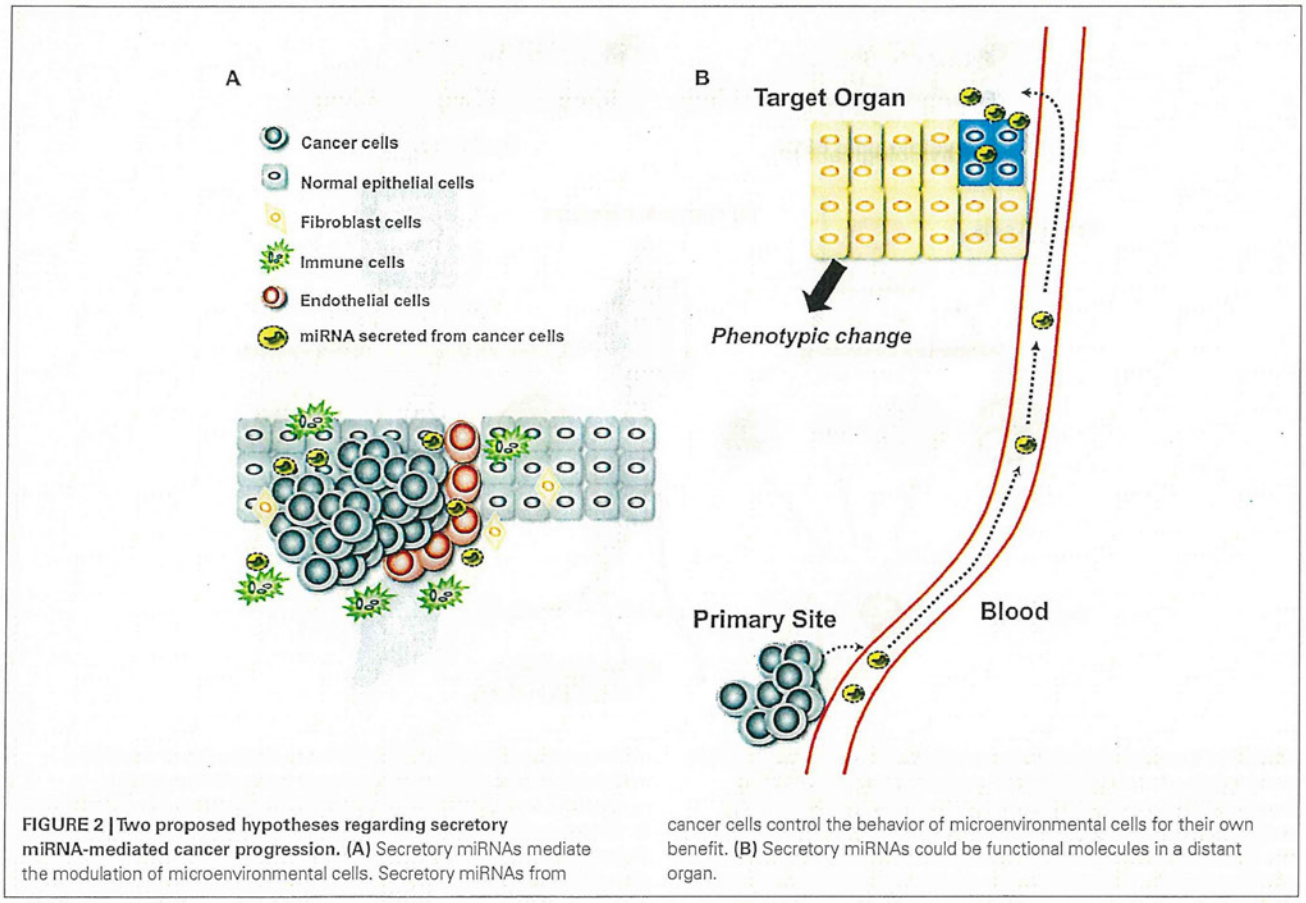

FIGURE 2 | Two proposed hypotheses regarding secretory miRNA-mediated cancer progression. (A) Secretory miRNAs mediate the modulation of microenvironmental cells. Secretory miRNAs from cancer cells control the behavior of microenvironmental cells for their own benefit. **(B)** Secretory miRNAs could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