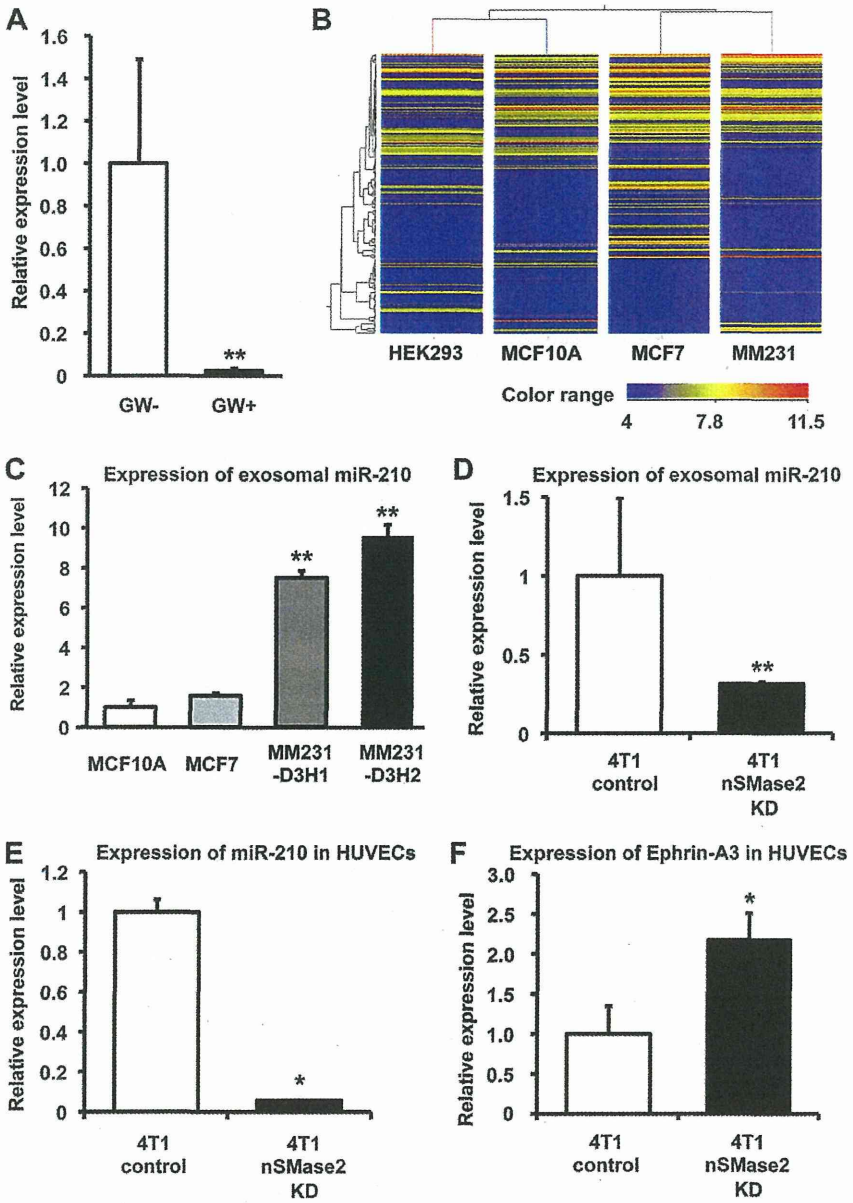

FIGURE 4. **Exosomal angiogenic miRNAs from cancer cells regulate angiogenesis in endothelial cells.** *A*, 4T1-siLuc cells were treated with 10 μM GW4869 at the start of the co-culture for a total of 48 h ($p < 0.001$). Each *error bar* is presented as the mean ± S.E. ($n = 3$). **, $p < 0.005$, as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

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

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

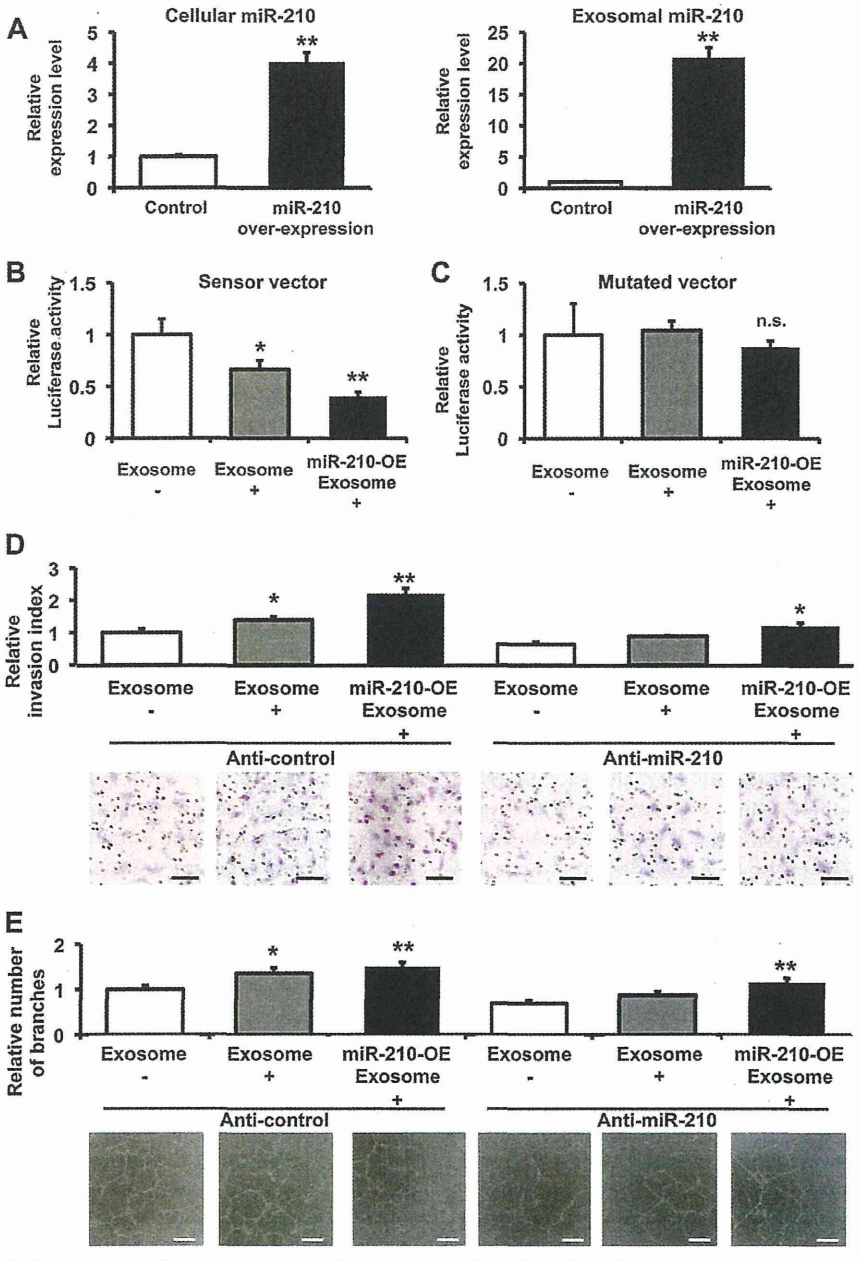

FIGURE 5. **Exosomal miR-210 from cancer cells enhanced the angiogenesis in endothelial cells.** *A*, the expression level of miR-210 in the cells (*left panel*) and exosome (*right panel*) from miR-210 overexpressing cells and control vector transfected cells. Each *error bar* is presented as the mean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

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

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

DISCUSSION

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

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

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

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

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

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Exosomal tumor-suppressive microRNAs as novel cancer therapy ☆ "Exocure" is another choice for cancer treatment

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ABSTRACT

MicroRNAs (miRNAs) act to fine-tune cellular responses in a variety of biological circumstances such as development, organogenesis, and homeostasis. The dysregulation of miRNA expression accelerates disease progression, including metabolic disease, immunological disease and cancer, through the gene network disorder. Therefore, understanding the miRNA maturation process may unravel the mechanisms of cancer malignancy; however, the life of miRNA has not been clarified. In this article, we summarize the recent findings regarding the novel forms of miRNA, especially secretory miRNAs, focusing on exosomal miRNAs. Recent research has revealed that exosomal miRNAs affect many aspects of physiological and pathological conditions, and may be useful as novel therapy. Here, we propose a method for the delivery of tumor-suppressive miRNAs to desired sites using exosomes, and we named this method "exocure".

Contents

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1. Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that are approximately 22 nt in length [1]. Although more than 1500 mature human miRNA sequences are currently listed in the miRNA database [2], the function of many has not been clarified. The maturation process of miRNAs includes several post-transcriptional processing steps [1]. First, the primary miRNA transcripts (primary miRNAs; pri-miRNA) are generated from the genomic sequence. These long transcripts are mainly transcribed by RNA polymerase II. Second, these transcripts are processed to precursor miRNA (pre-miRNA) in 60–110 nt fragments in the nucleus by Drosha, an RNase III enzyme. Then the pre-miRNAs are transported to the cytoplasm by exportin-5. Dicer, another RNase III enzyme, processes the pre-miRNA into double-stranded RNA, which is a mature miRNA of approximately 22 bp. The mature miRNA is composed of an RNA-induced silencing complex (RISC) and binds to the complementary sequence in the 3′ untranslated region (3′ UTR) of target mRNAs, resulting in the degradation of the mRNA and/or inhibition of protein translation [3]. This multistep processing is regulated by many factors such as epigenetic modifications of the genomic sequence and by transcription factors, RNase and RNA-binding proteins. If there is a misregulation of one of these processing components, the miRNA expression will be dysregulated, leading to disease progression such as cancer malignancy [4]. Recently, it was reported that miRNAs do not exist only in the cell but are also secreted outside of cells [5,6]. In this review, we will discuss recent reports that indicate that exosomes carrying "secretory miRNAs" mediate various biological phenomena. In addition, we propose the usage of an exosome-delivered tumor-suppressive miRNA in cancer therapy.

2. Tumor-suppressive miRNAs are novel anti-cancer agents

It is known that the downregulation of miRNA expression leads to cancer development through various mechanisms such as genomic deletions, amplifications, mutations, epigenetic silencing, the dysregulation of transcription factors that target specific miRNAs, or the inappropriate processing of miRNA precursors [4]. These downregulated miRNAs are thought to act as tumor suppressors. There are various types of tumor-suppressive miRNAs; however, little is known regarding their precise mechanism of action (Table 1).

2.1. let-7

The expression of let-7 miRNA is significantly downregulated in lung cancer, and the overexpression of let-7 in a lung adenocarcinoma cell line suppressed lung cancer cell growth in vitro through the downregulation of KRAS and HMGA2 [7–10]. Interestingly, let-7 miRNA expression was markedly reduced in breast cancer tumor-initiating cells [11], which have self-renewal ability and resistance to chemotherapy and radiotherapy, and the expression of let-7 miRNA increased after differentiation [12]. The re-expression of let-7 in breast cancer tumor-initiating cells reduced proliferation, mammosphere formation and metastasis in a xenograft model through the reduction of KRAS and/or HMGA2. Many downregulation mechanisms of let-7 have been reported [13–19]. Importantly, disruption of the let-7 precursor processing by LIN28 and LIN28B, which are reported as overexpressed in primary human tumors, human cancer cell lines and pluripotent stem cells, is essential for controlling proper miRNA expression [14].

2.2. miR-16

MiR-15a and miR-16-1 are deleted or downregulated in the majority of chronic lymphocytic leukemia (CLL) cells [20–22], and re-expression of these miRNAs induced apoptosis through the downregulation of BCL2. In addition, the miR-15a and miR-16 levels significantly decrease in advanced prostate tumor cells [23]. Takeshita et al. reported that the injection of miR-16 with atelocollagen (a highly purified type I collagen that possesses low immunogenicity and is produced by treating calf dermis with pepsin) via the tail vein of mice significantly inhibited the growth of prostate bone metastases in a therapeutic bone-metastasis model [24].

2.3. miR-143

The suppression of miR-143 expression has been reported in several human cancers, including colorectal, prostate, cervical and ovarian [25–30]. Induction of miR-143 expression in those cancer cells resulted in the inhibition of cell proliferation or the induction of apoptosis through the suppression of its target genes such as KRAS and ERK5. Furthermore, Osaki et al. showed that miR-143 was the most downregulated miRNA in metastatic human osteosarcoma cell lines relative to the parental cell lines, and transfection of miR-143 into metastatic human osteosarcoma cell lines significantly decreased cell invasiveness but not proliferation [31]. In addition, intravenous injection into mice of miR-143 significantly suppressed the formation of lung metastases from metastatic human osteosarcoma cell lines. Moreover, cells positive for MMP13, a target of miR-143 in osteosarcoma cells, was found in lung metastasis-positive cases but not in at least three cases with higher miR-143 expression levels and without metastases.

2.4. miR-22

Xu et al. demonstrated that miR-22 expression is upregulated in senescent human fibroblasts and epithelial cells. In contrast, its expression is downregulated in various cancer cell lines, and the overexpression of miR-22 in those cases induces growth suppression

Table 1
The list of typical tumor suppressive miRNAs.

microRNA	Type of cancer	Target gene	Phenotype	References
let-7	Lung cancer	KRAS	Inhibition of cell proliferation	[7–19]
	Breast cancer	HMGA2		
miR-16	Chronic lymphocytic leukemia	BCL2	Induction of apoptosis	[20–24]
	Prostate cancer	CCND1	Inhibition of cell proliferation	
		WNT3A		
miR-143	Ovarian cancer			[25–31]
	Prostate cancer	ERK5	Induction of apoptosis	
	Cervical cancer	KRAS	Inhibition of metastasis	
	Osteosarcoma		Inhibition of cell proliferation	
miR-22	Colorectal cancer	Sp1		[32]
	Breast cancer	CDK6	Induction of growth suppression	
		SIRT1	Induction of senescent phenotype	

Representative cases are shown in the "type of cancer" and "target gene".

and a senescent phenotype in human normal and cancer cells through the downregulation of CDK6, SIRT1, and Sp1 genes [32]. In addition, in vivo injection of miR-22 inhibits tumor growth and metastasis through the induction of senescence in inoculated breast cancer cell lines, suggesting that miR-22 can be used as a senescence inducer, and this approach may be a novel cancer treatment method.

Taken together, these findings prompted the idea that delivery of tumor-suppressive miRNAs that are downregulated in cancer cells may provide a therapeutic option in combination with other cancer treatments such as chemotherapy or antibody therapy.

3. Exosomal miRNAs are novel humoral factors for cell–cell communication

As described above, the expression levels of tumor-suppressive miRNAs are known to be downregulated in cancer cells. Therefore, restoring the expression of these miRNAs might lead to the suppression of cancer progression. However, the nucleic acid delivery method is the most significant problem for nucleic acid therapy [33,34]. In 2007, Valadi et al. found that miRNAs are contained inside exosomes [35]. This study showed the possibility that miRNAs are not only intracellular gene regulators but are also humoral factors, suggesting that miRNAs could act as tools for cell–cell communication. Exosomes are lipoprotein complexes including small-membrane vesicles of endocytic origin (30–100 nm) [35]. Exosomes are formed through the inward budding of endosomal membranes that give rise to intracellular multivesicular bodies (MVBs) that later fuse with the plasma membrane, releasing the exosomes to the extracellular space [36–39].

3.1. The exosomal miRNAs are functional in recipient cells

Following the report from Valadi et al., three reports showed the functionality of exosomal miRNAs (Table 2). Pegtel et al. showed that miRNAs encoded by the EB virus are secreted from EBV-infected B cells through exosomes. These miRNAs repress the EBV target immunoregulatory genes in primary EBV-associated lymphomas [40]. Zhang et al. reported that exosomes from human monocyte/macrophage cell lines deliver miR-150 into human microvascular endothelial cells, and the expression levels of c-Myb were downregulated in microvascular endothelial cells and enhance their cell migration [41]. We demonstrated that secreted miR-146a, whose expression is known to be downregulated in prostate cancer, was transferred from miR-146a-overproducing HEK293 cells to cancer cells, where it suppressed its target gene and led to cell growth inhibition [42].

3.2. Immune cells use exosomal miRNAs for cell–cell communication

After the publication of these studies, many researchers reported the function of exosomal miRNAs in a variety of physiological and pathological phenomena (Table 2). The antigen-driven unidirectional transfer of exosomal miRNAs from the T cell to antigen-presenting cells has been observed, and this transfer results in the downregulation of target gene expression in recipient cells [43]. Furthermore, dendritic cells (DCs) release exosomal miRNAs, which then transfer to acceptor DCs [44]. Interestingly, DCs release exosomes with different miRNAs depending on the maturation of the DCs. Taken together, these two papers regarding immune cells describe a novel mechanism of complex immune cell communication mediated by exosomal miRNAs. However, the transfer of miRNAs between cells is not limited to immune cells.

3.3. The function of exosomal miRNAs in cancer development

The exosomal miR-223 from tumor-associated macrophages is transported to breast cancer cells, supporting the idea that macrophages regulate the invasiveness of breast cancer cells through exosome-mediated delivery of oncogenic miRNAs [45]. In addition to breast cancer cells, exosomal miRNAs derived from hepatocellular carcinoma cells can be taken up by other cells and target transforming growth factor β activated kinase-1, resulting in the enhancement of transformed cell growth in recipient cells [46].

Recently, we showed that proliferation of a prostate carcinoma cell line was inhibited by the addition of the exosome fraction isolated from a non-cancerous prostate epithelial cell line [47]. These observations suggest that exosomal miRNAs derived from non-cancerous cells were transferred to cancerous cells and inhibit proliferation. Indeed, some sets of tumor-suppressive miRNAs such as miR-16, miR-205, and miR-143 were downregulated in prostate cancer cell lines at the cellular and extracellular levels. This observation supports the idea that secretory tumor-suppressive miRNAs are transferred from non-cancerous to cancerous cells in accordance with the miRNA concentration gradient. To examine in depth the contribution of secretory tumor-suppressive miRNAs in cancer initiation, we generated miR-143 overproducing HEK293 cells. We found that a prostate cancer cell line showed an approximately 50% decrease in proliferation through the suppression of the miR-143 target gene KRAS after the addition of an exosome derived from the miR-143-overproducing HEK293 cells. Importantly, the decrease was reversed by the transfection of anti-miR-143 in the prostate cancer cell line. These data indicate that the cell growth inhibition is attributable to the secretory miR-143 contained in the exosome of miR-143-overexpressing HEK293 cells [47].

Table 2
The list of exosomal miRNA mediating cell–cell communication both in basic research and research of therapeutic purpose.

Type of small RNA	Donor cells	Recipient cells	Phenotype	Target gene	References
EBV-miRNAs	LCL (EBV-transformed lymphoblastoid B cells)	MoDC (monocyte-derived dendritic cells)		CXCL11 LMP1	[40]
miR-150	THP-1 (human monocyte/macrophage cell line)	HMEC-1 (human microvascular endothelial cell)	Promote cell migration	c-Myb	[41]
miR-146a	HEK293	PC-3M	Growth inhibition	ROCK1	[42]
miR-335	J77 (T-cell line)	Raji (B-cell line)		SOX-4	[43]
miR-451 miR-148a	Bone marrow derived DCs	DC2.4 (mouse dentritic cell line)		[a]	[44]
miR-223	SKBR3 (human breast cancer cell line)	IL-4-activated macrophages (human monocyte-derived macrophages)	Promote invasion	Mef2c	[45]
Hep3B enriched miRNAs[b]	Hep3B (hepatocellular carcinoma)	Hep3B	Reduction in cell viability	TAK1	[46]
miR-143	PNT-2 HEK293	PC-3M	Growth inhibition	KRAS ERK5	[47]
miR-133a	H9c2 (rat cardiomyoblasts)	293FT		[a]	[60]
siRNA	Bone marrow derived DCs	Mouse brain		GAPDH	[48]
siRNA	Huh-7 (human hepatoma cell line)	Mouse hepatocyte		CD81	[49]

[a] The sensor vector, which is complementary sequence of miRNA, was used in this study.
[b] The target gene TAK1 was predicted by various types of miRNAs that were highly expressed in the exosome isolated from Hep3B.

4. Exosomes can be used as a small RNA delivery system

As described previously, resolving the issue of miRNA delivery is essential for cancer treatment by tumor-suppressive miRNAs. Therefore, it is natural to examine exosomal tumor-suppressive miRNAs for cancer treatment.

4.1. Exosomes can be used for siRNA delivery

Recently, the exosomal delivery of siRNAs to the mouse brain was reported [48]. In this report, self-derived DCs, which express the exosomal membrane protein Lamp2b fused with the neuron-specific RVG peptide 3, was used for the reduction of immunogenicity. Exosomal siRNA against GAPDH, which was loaded by electroporation, was intravenously injected, and it was delivered specifically to neurons, microglia, and oligodendrocytes in the brain, resulting in specific gene knockdown. In addition, using human hepatoma cells producing the viral entry receptor CD81 siRNA, siRNA delivery was confirmed, causing suppression of CD81 expression in mouse hepatocytes in vivo [49].

4.2. Exosomes can be used for miRNA delivery

As shown above, siRNA can be delivered by exosomes. It is plausible that tumor-suppressive miRNAs can be similarly delivered to cancer cells in vivo. As we have previously shown [47], the loading mechanism of miRNA and siRNA into exosomes is the same; therefore, this technique might be used for miRNA-mediated therapy. To address this possibility, we injected conditioned medium obtained from miR-143-overproducing or parental HEK293 cells into nude mice implanted with prostate cancer cells. The tumor expansion was restrained for 8 days with intratumor administration of miR-143-enriched conditioned medium. Consequently, the tumor masses shrank by approximately 0.5 fold on day 8. In addition, the expression of miR-143 target genes such as KRAS and ERK5 decreased following miR-143-transduced conditioned medium injections.

In our report, exogenously-transduced miR-143 did not suppress the proliferation of non-cancerous cells, suggesting that excessive amounts of tumor-suppressive miRNAs did not provide an additional growth inhibitory effect on normal cells, in which the expression of tumor-suppressive miRNAs is maintained at physiological levels [48].

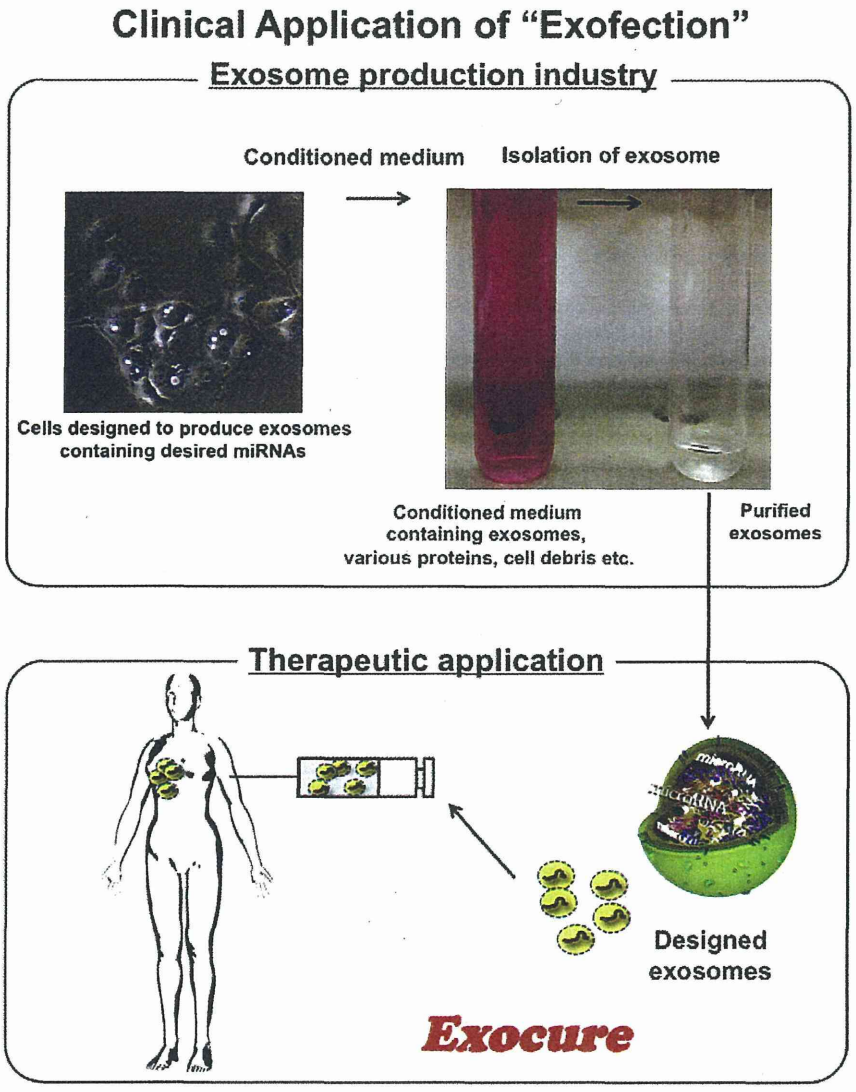

Fig. 1. A schematic explanation of "exocure". Exosome containing desired miRNAs, such as miR-16, miR-143 and so on, is produced by the "exosome production industry", and then these designed exosomes are delivered to cure the patient's disease. For this purpose, some of the issues which are showed in Fig. 2, need to be solved.

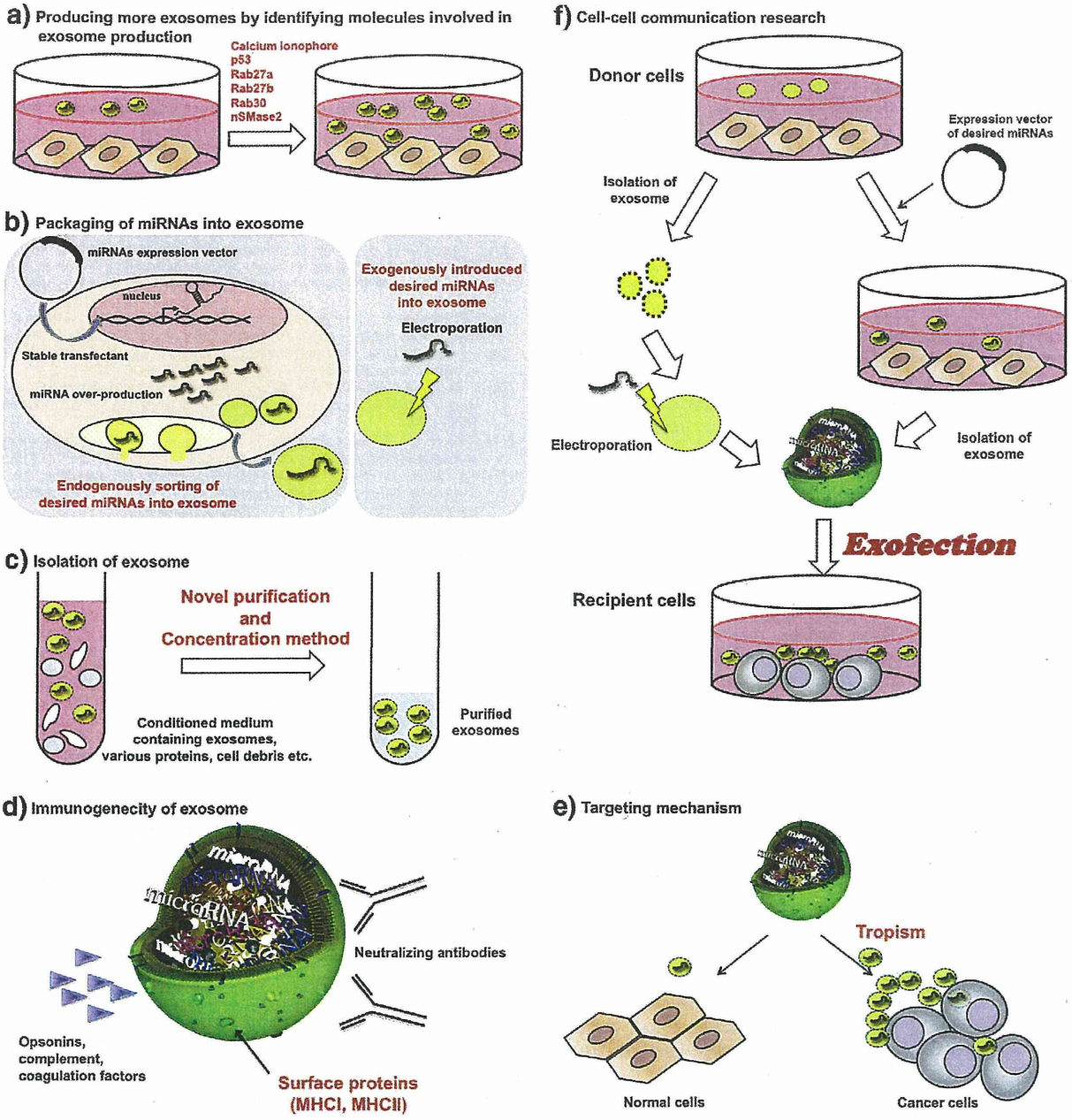

Fig. 2. *Issues for the clinical use of "exocure". A schematic explanation of how the novel treatment "exofection" was established and the issues to be solved.* To prepare exosomes containing tumor-suppressive miRNAs suitable for clinical usage, clarifying the mechanisms of exosome secretion is important. It is known that there are several molecules that regulate exosome secretion such as Rab27, Rab28, Rab30, nSMase2, p53 and calcium ionophore. Although the precise mechanism of exosome secretion has not yet been clarified, it is important to find the molecules that regulate exosome secretion (a). Currently, there are two kinds of methods to introduce desired miRNA into exosomes. One is to establish the cell line that stably overexpress desired miRNAs [47,49]. This enables us to obtain increased amount of desired miRNA in exosomes. The other one is exogenously introducing desired miRNAs using electroporation [48]. There are no knowledge about advantages and disadvantages in these two methods, moreover, the mechanism in which the tumor-suppressive miRNAs are sorted into exosomes is also unknown (b). These studies will enable us to obtain enough of the required exosomes for treatment. In addition, the establishment of exosome isolation methods is needed. The current, most popular isolation method, ultracentrifugation, is time-consuming and complicated, and its recovery rate is poor. Establishing more effective isolation methods is essential (c). Immunogenicity is an important factor to consider for the delivery of exosomes containing tumor-suppressive miRNAs. Exosomes are known to have reduced immunogenicity compared to other carriers such as viruses (d) [62]. Furthermore, the targeting mechanism of exosomes needs to be resolved (e). It has been known that exosomes have a tropism for target cells; however, the precise mechanism has not been found. Clarifying these five issues would enable us to establish the novel cancer treatment "exocure". The in vitro study of exosomal miRNAs might reveal many aspect of cross-kingdom research field, we suggest that this methods used for studying cell–cell communication be named "exofection" (f).

In addition, no overt side effects were observed in exosome-mediated gene delivery in vivo by dendritic cell-derived exosome [48]. Taken together, these reports suggest that exosomal tumor-suppressive miRNA therapy does not have serious side-effects [47,48].

5. Perspectives

In this review, we have summarized the knowledge regarding exosomal miRNAs for cancer therapy. We want to emphasize that

exosomal tumor-suppressive miRNAs are promising molecules for cancer therapy, (Fig. 1) although their use may have several difficulties (Fig. 2). First, the exosome must be abundant or highly enriched in order to utilize in therapy. It is known that exosome production was regulated by several molecules such as nSMase2, Rab27a, Rab27b, Rab35, p53 and calcium ionophore; however, the precise production mechanisms have not been clarified [50–59]. If the exosome production mechanism was clarified, we could develop "exosome-producing cells" by cell engineering. Second, the methods introducing desired tumor suppressive miRNAs into exosomes should be considered. There are two possible methods to introduce desired miRNAs into exosomes. One is the overexpression of desired miRNAs in the cells, resulting in the increased amount of miRNAs inside exosomes [42,47]. The other one is exogenously introducing miRNA into exosomes by electroporation [48]. Only few reports employed these methods and more studies need to be carried out for these methods to recognize more effective methods. Thirdly, methods for isolation of exosomes from conditioned medium need to be developed. The current, most popular isolation method, ultracentrifugation, is time-consuming and complicated, and its recovery rate is poor. Establishing more effective isolation methods is essential. Moreover, the exosome-producing cells need to be carefully chosen. Because of the tropism against the target cancer cells, we need to understand the mechanism of exosome uptake. In addition, the immunogenicity of exosomes is poorly understood. Resolving these issues may result in a safe and cost-effective exosome delivery method. Last, the functions and mechanisms of tumor-suppressive miRNAs need to be clarified. The choice of target miRNAs may aid in the decisions regarding cancer therapy such as radiotherapy and/or chemotherapy. Because the exosome is an ideal and promising delivery material for small RNA therapy, we suggest that this method be named "exocure" (Fig. 1).

In addition, this method can also be utilized for research of cell–cell communication. As shown in Table 2, these reports regarding the exosomal miRNAs open up a novel research field for the cell–cell communication. Surprisingly, recent reports showed that exogenous plant miRNAs can be found in the blood sera of animals and this plant miRNAs were considered to exist inside exosomes, suggesting that plant miRNAs can regulate the expression of target genes in mammals [61]. Although more studies need to be done about plant miRNAs in human body fluids, studying the exosomal miRNAs might unveil the mystery of this cross-kingdom gene regulation. To study the precise function of exosomal miRNAs, the methods that we proposed in Fig. 2 can be used. The in vitro study of exosomal miRNAs might reveal many aspect of cross-kingdom research field, we suggest that this methods used for studying cell–cell communication be named "exofection".

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