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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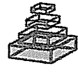

Trash or Treasure: extracellular microRNAs and cell-to-cell communication

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

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

INTRODUCTION

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

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

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

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

miRNAs IN EXTRACELLULAR VESICLES OR NON-VESICLE ASSOCIATED miRNAs

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

miR-210

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

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

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

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

EBV miRNAs

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

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

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

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

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

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

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

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

miR-21

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

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

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

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

miR-126

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

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

miR-451

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

miR-223

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

miR-150

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

SUMMARY AND PERSPECTIVES

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

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

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

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

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

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

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

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

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

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INTRODUCTION

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

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

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

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

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

RESULTS

GE11- and EGF-positive exosomes

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

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using the transmembrane domain of platelet-derived growth factor receptor (Figure 1a). We transfected human embryonic kidney cell line 293 (HEK293) cells with pDisplay encoding GE11 or EGF, and then cloned cells that were stably expressing the constructs. Exosomes were purified from culture supernatants using an ultracentrifugation protocol (see Materials and Methods section).

We then examined the expression of GE11 or EGF in exosomes using anti-hemagglutinin (HA) antibodies and western blot analysis, which revealed bands of the predicted sizes (Figure 1b). In addition, fluorescence-activated cell sorting (FACS) confirmed the presence of GE11 and EGF on the outer membranes of exosomes bound to latex beads, and these complexes were recognized by anti-Myc-tag antibodies (Figure 1c). CD81 was used as a positive control for exosomes. Myc-tag expression was observed more frequently in EGF-positive (73.8%) and GE11-positive (66.2%) exosomes than in vector control (14.3%) exosomes. These data indicated that GE11 or EGF was present on the exosomal membranes. Additionally, immunogold staining with anti-HA antibodies showed that 15.3% and 21.2% of the exosomes were positive for GE11 and EGF, respectively, and no notable morphologic abnormalities were observed in the modified exosomes (Figure 1d).

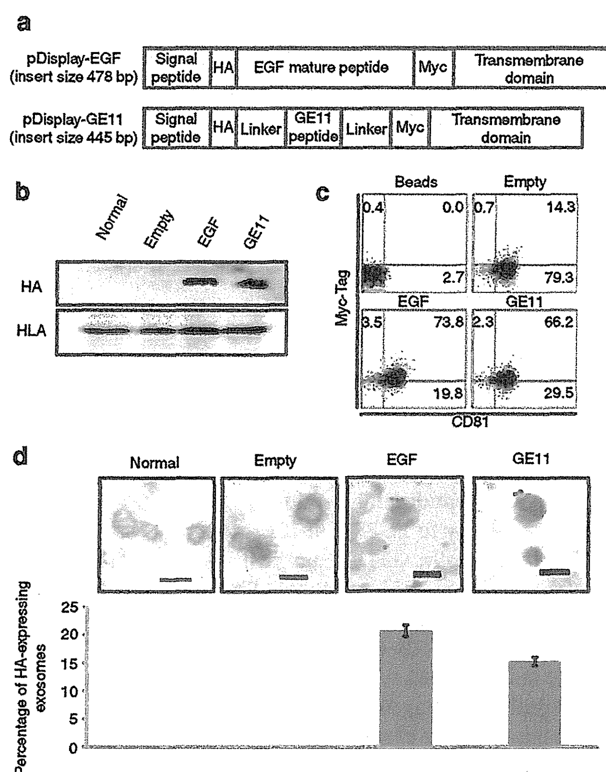

Figure 1 Epidermal growth factor receptor (EGFR) ligands on the outer surfaces of the exosomes. (a) Diagrams of the modified epidermal growth factor (EGF) and GE11 proteins. Signal peptide, Igκ-chain leader sequence; HA, hemagglutinin epitope tag (YPYDVPDYA); Linker, (GGGGS) 3; Myc, Myc epitope (EEKLISEEDL); platelet-derived growth factor receptor (PDGFR) transmembrane domain, transmembrane domain from platelet-derived growth factor receptor. (b) Western blots of HA-tagged constructs in exosomes obtained from culture supernatants of human embryonic kidney cell line 293 (HEK293) cells that had been transfected with pDisplay encoding EGF or GE11. The quality of each exosome preparation was confirmed by hybridization with antihuman leukocyte antigen (HLA) antibodies. (c) For flow cytometry, exosomes from transfected HEK293 cells were incubated with latex beads and stained with anti-Myc tag antibodies. Tetraspanin CD81 was used as a positive control for the exosomes. (d) Immunoelectron microscopy showed that HA-tagged constructs were present on exosomes purified from the supernatants of cells transfected with pDisplay encoding EGF or GE11. Bars = 100 nm. The percentages of HA-positive exosomes are indicated in the graph. Data are expressed as means ± SD.

EGFR-dependent uptake of modified exosomes *in vitro*

We next examined whether the GE11- or EGF-positive exosomes derived from HEK293 cells bound to recipient cells in an EGFR-dependent manner. We first evaluated EGFR expression in three human breast cancer cell lines. HCC70 cells showed higher EGFR expression levels than HCC1954 and MCF-7 cells (Figure 2a). To examine whether GE11- or EGF-positive exosomes were taken up by recipient cells, exosomes were labeled with PKH67 dye (green) and added to cultures of HCC70 cells (Figure 2b). EGF- and GE11-positive exosomes more efficiently bound to HCC70 cells than HCC1954 or MCF-7 cells. Binding appeared to reflect EGFR expression levels (Figure 2b). Exosomes did not bind to cell surface membranes when the samples were incubated at 4 °C, suggesting that the cells had to be biologically active (Figure 2b). Confocal laser-scanning microscopy demonstrated that the exosomes were internalized by the cells (Figure 2c). To confirm that EGF- and GE11-positive exosomes were taken up by an EGFR-dependent mechanism, we performed assays using breast cancer cell lines with different levels of EGFR expression. First, we prepared MCF-7 cells expressing high levels of EGFR using a retroviral vector (Figure 2d) and examined uptake of EGF- and GE11-positive exosomes (Figure 2e). EGF- and GE11-positive exosomes showed high affinities for these MCF-7 cells compared with cells infected with empty vector or untreated cells. Next, we prepared HCC70 cells in which EGFR expression was knocked down using siRNA. Three days post-transfection, EGFR expression was markedly reduced, and we examined exosome uptake using fluorescence-activated cell sorting analysis (Figure 2f). Because the cells proliferated following transfection of EGFR siRNA, relatively low levels of siRNA were detected in PKH67-labeled exosomes compared with the experiment shown in Figure 2b. Of note, however, siRNA levels decreased to near background levels in HCC70 cells in which EGFR expression was reduced compared with cells expressing high levels of EGFR or cells transfected with nontarget siRNA.

To assess whether EGF- or GE11-positive exosomes affected cell growth *in vitro*, we performed cell proliferation assays using HCC70 cells. EGF-positive exosomes promoted cell proliferation, whereas no effect was noted with control or GE11-positive exosomes (**Supplementary Figure S1**). These experiments suggested that, unlike EGF-positive exosomes, GE11-positive exosomes do not stimulate EGFR signaling. Thus, GE11-positive exosomes may provide a more suitable drug delivery system than EGF-positive exosomes.

GE11-positive exosomes are functional *in vitro*

Our results indicated that the modified exosomes were taken up into recipient cells. Next, we investigated exosome-mediated delivery of siRNA or miRNA *in vivo*, including the effects of the exogenous siRNA or miRNA in the recipient cells. We first

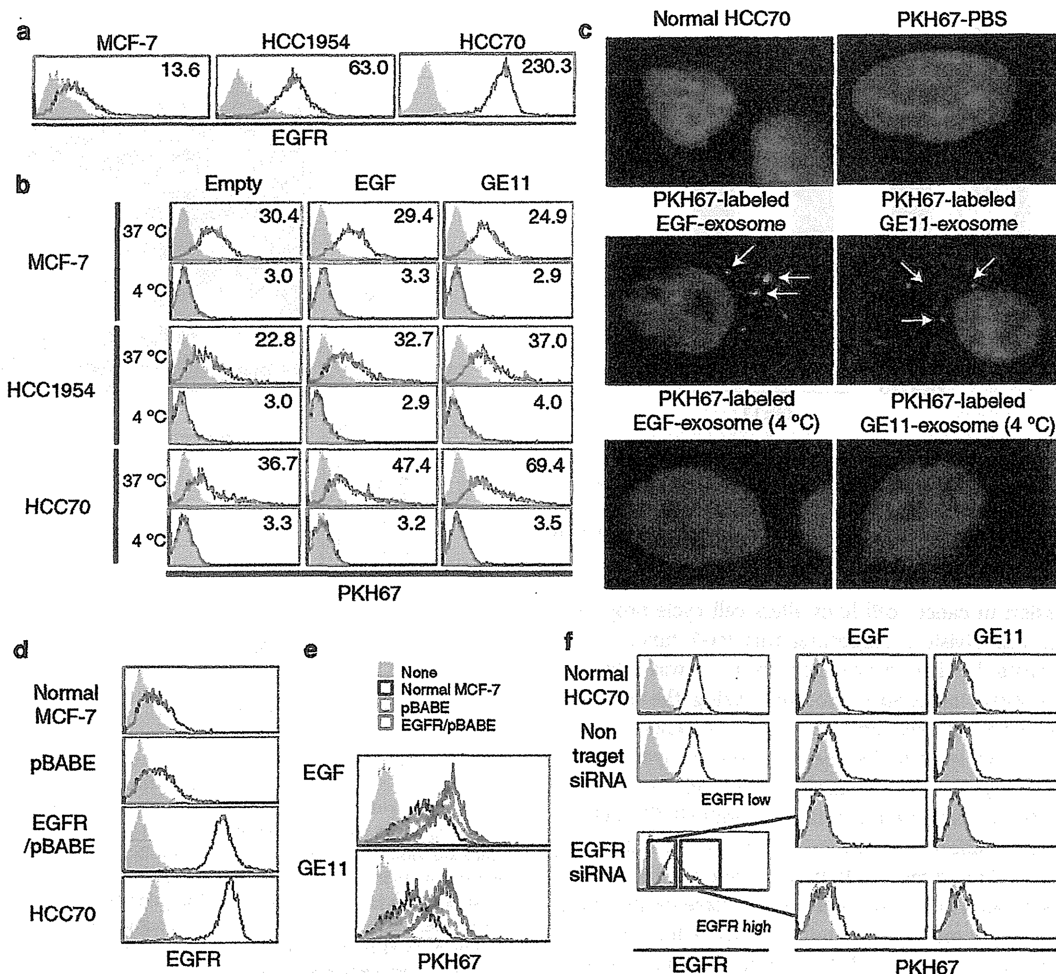

Figure 2 Uptake of epidermal growth factor (EGF)- and GE11-positive exosomes by breast cancer cell lines. (**a**) Flow cytometric analysis of epidermal growth factor receptor (EGFR) expression on HCC70, HCC1954, and MCF-7 breast cancer cells. (**b**) Uptake of fluorescently labeled exosomes by the breast cancer cell lines was detected using flow cytometry. PKH67-labeled exosomes were incubated with the breast cancer cell lines at 37 °C or 4 °C for 4 hours. The degree of uptake was relatively low at 4 °C. (**c**) Intracellular PKH67-labeled exosomes were detected in HCC70 cells (arrows) using confocal fluorescence microscopy. (**d**) Flow cytometric analysis of EGFR expression on MCF-7 cells, which were stably infected with retrovirus expressing EGFR. (**e**) Uptake of PKH67-labeled EGF- and GE11-positive exosomes was compared using MCF-7 cells expressing high levels of EGFR and control cells. (**f**) Uptake of PKH67-labeled EGF- and GE11-positive exosomes was compared among $EGFR^{Low}$ HCC70, $EGFR^{High}$, and control cells.

transfected HEK293 cells with luciferase-specific siRNA and purified loaded exosomes. Then, we added the exosomes to culture medium containing luciferase-expressing HCC70 cells. After 24 hours, we measured luciferase activities and found that GE11-positive exosomes containing luciferase-specific siRNA reduced luciferase activity in the cells (Figure 3). These exosomes likely contained only a fraction of the transfected siRNA from the exosome-secreting cells. Nevertheless, the encapsulated siRNA significantly inhibited expression of the target luciferase gene.

GE11-positive exosomes bind tumor cells *in vivo*

We then examined whether GE11-positive exosomes specifically bind to tumors *in vivo*. Luciferase-expressing HCC70 cells were transplanted into the mammary fat pads of $RAG2^{-/-}$ mice. GE11-positive and control exosomes were labeled with XenoLight DiR and intravenously injected into tumor-bearing $RAG2^{-/-}$ mice via the tail vein. Twenty-four hours later, the locations of the exosomes were monitored using an *in vivo* imaging system (IVIS). Signals from the GE11-positive exosomes were detected in the xenograft tumors, whereas little signal was detected in experiments using control exosomes (Figure 4a). We also counted the exosomes and observed that, compared with control exosomes, three times as many GE11-positive exosomes reached the tumor (Figure 4b). In addition, we did not histologically detect any major organ damage in the injected mice (**Supplementary Figure S2**). These data indicated that GE11-positive exosomes may facilitate the delivery of therapeutic molecules to EGFR-expressing tumors *in vivo*.

GE11-positive exosomes containing let-7 inhibit tumor development *in vivo*

We next investigated the delivery of miRNA to tumors using the GE11-positive exosomes. We used let-7a because elevated

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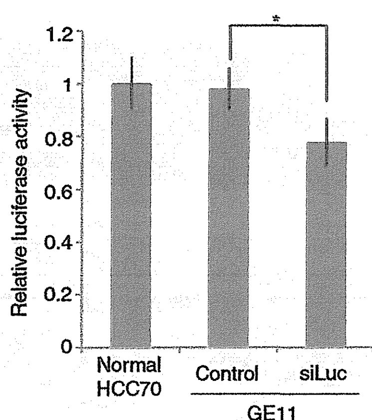

Figure 3 The activity of encapsulated small-interfering RNA (siRNA) in luciferase assays. Luciferase-specific siRNA (siLuc) was encapsulated in exosomes, which were incubated for 48 hours with HCC70 cells stably expressing firefly luciferase. Data are expressed as means ± SD. $n = 3$; $*P < 0.05$.

let-7 expression in cancer cell lines alters cell cycle progression and reduces cell division, suggesting that let-7 functions as a tumor suppressor.[14,15] Let-7a or control miRNA was introduced into GE11-positive or control exosomes using the lipofection method and HEK293 cells, and the amount of loaded miRNA was determined in quantitative real-time reverse transcription-PCRs (Figure 5a). To measure tumor growth in RAG2$^{-/-}$ mice, we prepared tumor-bearing mice with xenograft HCC70 cells that stably expressed luciferase. To analyze tumor growth and development, we injected the tumors with luciferin and monitored signal emission using an IVIS. Exosomes were intravenously injected into tumor-bearing mice via the tail vein. After four injections, tumor growth was measured. Let-7a–containing GE11-positive exosomes markedly suppressed tumor growth (Figure 5b and c; $n = 6$, $P < 0.01$). Several studies have reported that let-7a inhibits tumor development by reducing expression levels of HMGA2 or members of the RAS family (K-RAS, H-RAS, N-RAS).[16] We examined the expression of these genes in let-7a–transfected HCC70 cells using real-time reverse transcription-PCR analysis, immunoblotting, and immunostaining (**Supplementary Figure S3a–c**). Furthermore, we immunohistochemically assessed the expression of HMGA2 and RAS family members in xenograft tumors (**Supplementary Figure S3d**). Let-7a did not affect the expression of HMGA2 or RAS family members *in vivo* or *in vitro*. Consistent with previous reports, however, let-7a potently inhibited the expression of HMGA2 mRNA in A549 lung adenocarcinoma cells[17] (**Supplementary Figure S3a**). These data indicated that let-7a inhibits tumor development via previously unidentified or uncharacterized genes in HCC70 breast cancer cells. Taken together, these findings indicated that GE11-positive exosomes are a promising vehicle for delivering drugs to EGFR-expressing tumors.

DISCUSSION

Although miRNA is a promising anticancer therapeutic modality, the clinical use of these RNA molecules has been hampered by a lack of malignant tissue–specific delivery systems. In the present study, we showed that exosomes can be used to efficiently deliver antitumor miRNA to cancer tissues *in vivo*.

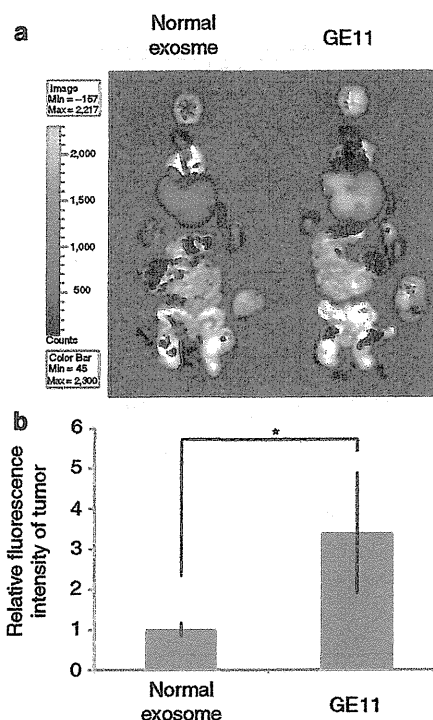

Figure 4 Migration of GE11-positive exosomes to tumor tissues characterized by high levels of epidermal growth factor receptor (EGFR) expression. (a) Exosomes labeled with XenoLight DiR (near-infrared) were intravenously injected (4 μg of purified exosomes) into mice bearing transplanted HCC70 cells. Brain, heart, spleen, liver, lung, kidney, small intestine, colon, and tumor tissues were harvested 24 hours postinjection for *ex vivo* imaging. The migration of fluorescently labeled exosomes was detected with an *in vivo* imaging system (IVIS). (b) The intensity of fluorescent signals from the tumor was measured using an IVIS. Data are expressed as means ± SD. $n = 5$; $*P < 0.05$.

A number of nanocarriers using various materials have been developed for drug delivery systems. Polyethylene glycol–coated liposomes, which are frequently used as carriers for *in vivo* drug delivery, benefit from easy preparation techniques, acceptable toxicity profiles, and a lack of clearance by the reticuloendothelial system. Liposomes, however, have several drawbacks, including the efficiency of targeting and issues associated with accelerated blood clearance.[18] Although exosomes and liposomes have similar phospholipid bilayers, exosomes consist of only biogenic substances. The potential of exosomes as drug delivery carriers, however, is largely unknown. Adding appropriate targeting molecules can cause exosomes to accumulate at sites of disease *in vivo* (ref. 7 and Figure 4). Thus, the biocompatibility and toxicity profiles of exosomes, which notably are natural carriers of miRNA *in vivo*, support their application in drug delivery systems.

To use exosomes clinically, however, further studies are needed to resolve several issues. For instance, therapeutic exosomes should not be quickly cleared by the reticuloendothelial system. In this study, when fluorescently labeled exosomes were injected into the mice tail vein, many exosomes accumulated

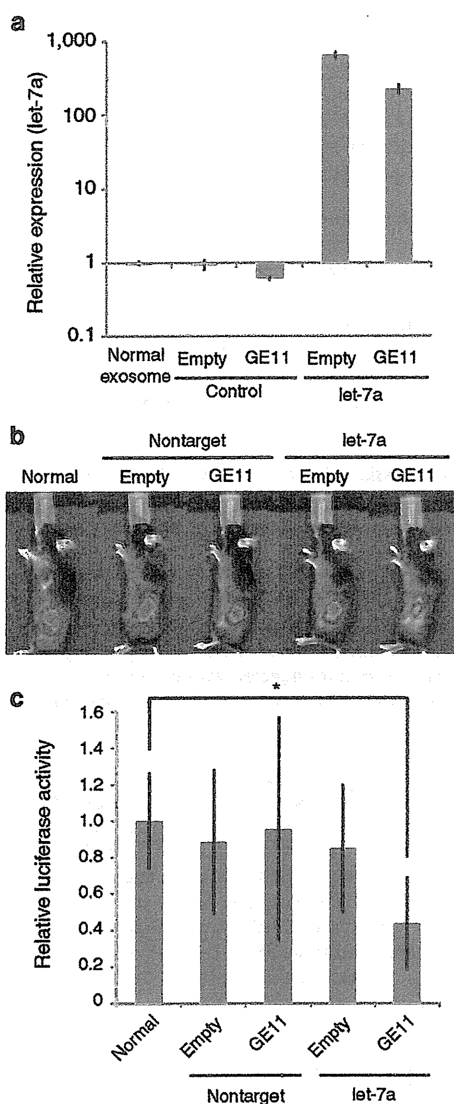

Figure 5 Inhibition of breast cancer development *in vivo* using GE11-positive exosomes containing let-7a. Human embryonic kidney cell line 293 (HEK293) cells expressing GE11 were transfected with synthetic let-7a. Exosomes containing let-7a were purified from culture supernatants and intravenously injected (1 µg of purified exosomes, once per week for 4 weeks) into mice bearing luciferase-expressing HCC70 cells. (**a**) Let-7a levels in the purified exosomes were measured using quantitative PCRs. Data are expressed as means ± SD. $n = 3$. (**b**) Representative images of tumors 4 weeks postinjection are shown. (**c**) Luciferase signals from the tumors were measured using an *in vivo* imaging system (IVIS). Data are expressed as means ± SD. $n = 5$; $^{*}P < 0.05$.

in the liver 24 hours after the injection (Figure 4a). Thus, some modifications are required to avoid normal clearance mechanisms.

This type of delivery strategy requires that the miRNA or siRNA can be efficiently introduced into the exosomes. We transfected donor cells with miRNA and purified exosomes from the culture supernatant. A previous report described electroporation protocols for loading siRNA into exosomes.[8] We, however, were unable to use these methods to load our exosomes with miRNA. The differences in the results may have been caused by the different cell types that were used in the two studies.

The composition of exosomes appears to differ depending on the source tissue or cell type. For instance, major histocompatibility complex class II molecules are enriched in exosomes from B lymphocytes, dendritic cells, mast cells, and intestinal epithelial cells, whereas higher levels of growth factors and their receptors are found in exosomes released from cancer cells.[19] For allogeneic exosome therapy, the presence of major histocompatibility complex proteins is problematic owing to potential immune responses. Therefore, the appropriate selection of donor cells for exosome production is a key factor for potential clinical applications of exosome-based therapies.

In conclusion, exosomes targeted to tumors may allow systemic administration of miRNA as cancer therapy. Technologic improvements that enhance exosome production and reduce immunogenicity should be explored to further develop this drug delivery approach.

MATERIALS AND METHODS

Plasmids transfection and retrovirus infection. The pDisplay vector was purchased from Invitrogen (Carlsbad, CA). Sequence encoding GE11 peptide (YHWYGYTPQNVI) with a flexible peptide linker (GGGGS)3 or mature EGF (53 amino acids, GenBank accession number P01133) was directly fused into pDisplay (Figure 1a). HEK293 cells were transfected with pDisplay encoding GE11 or EGF using FuGENE HD transfection reagent (Promega, Madison, WI) and selected with Geneticin (Invitrogen).

EGFR retroviral vector was purchased from Addgene (Cambridge, MA). Viral supernatants were produced by transient transfection of GP2-293T cells with a packaging plasmid (pVSV-G) according to the manufacturer's instructions (Clontech, Mountain View, CA). MCF-7 cells were infected with viral supernatants using polybrene at a final concentration of 8 µg/ml.

Cell culture and small RNA transfection. Breast cancer cell lines (HCC70, HCC1954, and MCF-7) and a HEK293 were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured according to the manufacturer's instructions. HCC70 cells express firefly luciferase, as previously described.[20] miRNA and siRNA used in this study were as follows: has-let-7a sense (5′-UGAGGUAGUAGGUUGUAUAGUU-3′) and antisense (5′-CUAUACAAUCUACUGUCUUUC-3′); nontarget control miRNA sense (5′-AUCCGCGCGAUAGCACGUAUU-3′) and antisense (5′-UACGUACUAUCGCGCGGAUUU-3′); EGFR-specific siRNA sense (5′-GUGAGGUGGUCCUUGGGAATT-3′) and antisense (5′-UUCCCAAGGACCACCUCACTT-3′); luciferase-specific siRNA sense (5′-CUUACGCUGAGUACUUCGATT-3′) and antisense (5′-UCGAAGUACUCAGCGUAAGTT-3′); and nontarget control siRNA sense (5′-AUCCGCGCGAUAGCACGUATT-3′) and antisense 5′-UACGUACUAUCGCGCGGAUTT-3′). Oligonucleotides were transfected into cells using HiPerFect reagent (final concentration, 50 nmol/l; Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Western blot analysis. Western blot analysis was performed as previously described.[21] Exosome samples were lysed in sodium dodecyl sulfate loading buffer. After boiling, equal amounts (4 µg) of the proteins were electrophoresed on 15% sodium dodecyl sulfate-polyacrylamide gels and transferred to Immobilon membranes (Millipore, Bedford, MA) using semidry blotting. Then, using standard techniques, the membranes were probed with antibodies, including anti-HA (HA7) (Sigma-Aldrich, St Louis, MO) and anti-HLA-A/B/C (H-300) (Santa Cruz Biotechnology, Santa Cruz, CA). Labeling was visualized using Immobilon Western chemiluminescent

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horseradish peroxidase substrate (Millipore) and signals were examined on an LAS-3000 mini system (Fujifilm, Tokyo, Japan).

RNA isolation and quantitative real-time reverse transcription-PCRs. RNA was isolated from exosomes using Isogen reagent (Nippon Gene, Osaka, Japan) according to the manufacturer's instructions. miRNA levels were quantified using TaqMan miRNA assays (Applied Biosystems, Carlsbad, CA). Copy numbers were calculated based on a standard curve created using synthetic RNA. miRNA levels were normalized based on has-miR-16 levels. Quantitative PCRs were run on a Stratagene MX3000P thermocycler and analyzed with MxPro (Agilent Technologies, Santa Clara, CA).

Preparation of exosomes. Exosomes were prepared from HEK293 cells that had been cultured for 48 hours in Dulbecco's modified eagle medium supplemented with 1% GlutaMax (Invitrogen). Cell supernatants were subjected to differential centrifugation. To eliminate cellular debris, samples were centrifuged at 2,000*g* for 20 minutes and 10,000*g* for 30 minutes. Exosomes were pelleted via ultracentrifugation at 120,000*g* for 70 minutes and washed once in phosphate-buffered saline. Protein content in the exosomes was measured using a Protein Assay Rapid Kit (Wako Pure Chemicals, Osaka, Japan). The average exosome yield was 69.2 μg from 100 ml ($2–5 \times 10^7$ cells) of culture supernatant ($n = 8$).

Flow cytometry. For fluorescence-activated cell sorting, exosomes from HEK293 cells were adsorbed onto 4-μm aldehyde-sulphate latex beads (Interfacial Dynamics, Tualatin, OR), incubated with Alexa Fluor 488–conjugated anti-Myc tag antibodies (Millipore, Temecula, CA) or allophycocyanin-conjugated anti-CD81 antibodies (BD Pharmingen, San Jose, CA), and analyzed on a FACSCalibur system (Becton Dickinson, San Diego, CA).

Immunoelectron microscopy. Purified exosomes from HEK293 cells were fixed in 2% paraformaldehyde and loaded onto Formvar-coated Ni electron microscopy grids. The samples were incubated overnight at 4 °C with anti-HA antibodies (Sigma-Aldrich) followed by 1 hour at room temperature with anti-mouse immunoglobulin G conjugated with 15-nm colloidal gold particles. The samples then were fixed in 2% glutaraldehyde, stained with 1% phosphotungstic acid, air-dried, and analyzed using a Hitachi H-7000 electron microscope (Hitachi High-Technologies, Tokyo, Japan). Exosomes positive or negative for gold particles were counted in 10 grids (~1,000–2,000 exosomes).

Coculture of PKH67-labeled exosomes and breast cancer cell lines. Exosomes were stained with green PKH67 fluorescent dye (Sigma-Aldrich). After staining, exosomes were washed with phosphate-buffered saline and centrifuged at 120,000*g* for 70 minutes. One microgram of PKH67-labeled exosomes was incubated with 1×10^5 breast cancer cells at 37 °C or 4 °C for 4 hours. The uptake of PKH67-labeled exosomes was analyzed using flow cytometry and confocal fluorescence microscopy.

Administration of let-7a–containing exosomes in a human tumor xenograft model. Luciferase-expressing HCC70 cells (2×10^6) were injected subcutaneously into the mammary fat pads of 5-week-old RAG2$^{-/-}$ mice. Four weeks after transplantation, tumors were sized using an IVIS (Xenogen, Hopkinton, MA). HEK293 cells expressing GE11 were transfected with synthetic let-7a. Let-7a–containing exosomes were purified from culture supernatants and intravenously injected (1 μg of purified exosomes, once per week for 4 weeks) into mice with transplanted luciferase-expressing HCC70 cells. Let-7a levels in the exosome samples were evaluated using TaqMan miRNA assays and real-time PCRs. Mice were handled according to the Ethical Guidelines of our institution. All experiments were approved by the Committee for Animal Research at our institution.

In vivo *imaging of fluorescently labeled exosomes.* A stock solution of the lipophilic near-infrared dye XenoLight DiR (Caliper Life Sciences, Hopkinton, MA) was prepared in ethanol. A 300-μmol/l working solution was prepared in diluent-C solution (Sigma-Aldrich). Exosomes isolated from culture supernatant–derived HEK293 cells were incubated with 2 μmol/l DiR for 30 minutes. The exosomes were then washed with 10 ml of phosphate-buffered saline, subjected to ultracentrifugation, and injected intravenously into RAG2$^{-/-}$ mice (4 μg of exosomes/mouse). Migration of fluorescently labeled exosomes in murine organs was detected using an IVIS 24 hours postinjection.

In vivo *imaging of xenograft tumors.* Mice were anesthetized via isoflurane inhalation, and intraperitoneally injected with 100 μl of 7.5 mg/ml luciferin solution (Promega). Bioluminescence imaging was initiated with an IVIS (Xenogen) 10 minutes postinjection. The region of interest was defined manually, and bioluminescence data are expressed as photon flux values (photons/s/cm^2/steradian). Background photon flux was defined using an area of the tumor that did not receive an intraperitoneal injection of luciferin. All bioluminescence data were collected and analyzed using an IVIS.

Statistical analysis. Differences were statistically evaluated using one-way analysis of variance followed by the Fisher protected least significant difference test. *P* values <0.05 were defined as statistically significant.

SUPPLEMENTARY MATERIAL

Figure S1. Analysis of cell viability based on assays with (4, 5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide.
Figure S2. Hematoxylin and eosin staining of lung, liver, spleen, and kidney tissues from mice injected with exosomes.
Figure S3. Expression analysis of the let-7 target genes.

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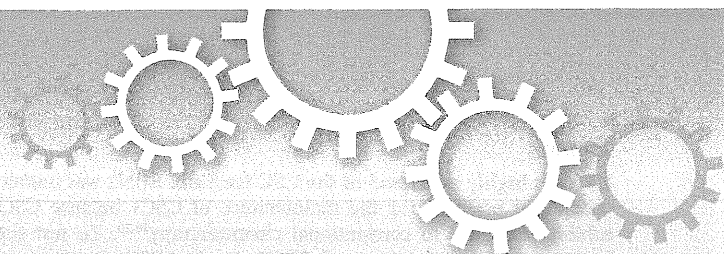

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Ribophorin II regulates breast tumor initiation and metastasis through the functional suppression of GSK3β

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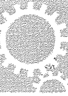
Mutant p53 (mtp53) gain of function (GOF) contributes to various aspects of tumor progression including cancer stem cell (CSC) property acquisition. A key factor of GOF is stabilization and accumulation of mtp53. However, the precise molecular mechanism of the mtp53 oncogenic activity remains unclear. Here, we show that ribophorin II (RPN2) regulates CSC properties through the stabilization of mtp53 (R280K and del126-133) in breast cancer. RPN2 stabilized mtp53 by inactivation of glycogen synthase kinase-3β (GSK3β) which suppresses Snail, a master regulator of epithelial to mesenchymal transition. RPN2 knockdown promoted GSK3β-mediated suppression of heat shock proteins that are essential for mtp53 stabilization. Furthermore, our study reveals that high expression of RPN2 and concomitant accumulation of mtp53 were associated with cancer tissues in a small cohort of metastatic breast cancer patients. These findings elucidate a molecular mechanism for mtp53 stabilization and suggest that RPN2 could be a promising target for anti-CSC therapy.

Recent studies show that some p53 mutations result in the loss of tumor-suppressing function (LOF) by the mutant allele and *trans*-dominant inactivation of the remaining wtp53[1]. Importantly, the mutant p53 (mtp53) contributes to tumor progression. This mechanism is referred to as mtp53 gain of function (GOF). A key property of GOF is the stabilization and accumulation of mtp53[2]. The mtp53 protein is rescued from degradation and contributes to malignant phenotypes such as invasion and metastasis or genomic instability by binding and inactivating p63 and Mre11, respectively[3–5]. In contrast to wild-type p53 (wtp53), the mtp53 protein adopts an aberrant conformation. Mtp53 forms stable complexes with heat shock proteins HSP90 and HSP70, MDM2, and the carboxyl terminus of HSP70-interacting protein (CHIP), which prevent mtp53 misfolding and aggregation[6,7]. In malignant cancer cells, the HSP90 and HSP70 chaperone machinery is upregulated and activated to protect mutated and overexpressed oncoproteins from degradation[8,9]. While several studies succeeded in identifying the molecular mechanisms that regulate mtp53 stability[6,7] and the small molecules that induce mtp53 destabilization[6,10], the mechanism that leads to mtp53 stabilization is not yet fully understood.

In breast cancer, p53 LOF or mutation induces epithelial to mesenchymal transition (EMT)[11], which contributes to cancer progression and metastasis[12,13]. Several studies show a link between EMT and the acquisition of CSC properties[11,14]. Ectopic expression of EMT regulators, such as Twist and Snail, or shRNA-mediated knockdown of E-cadherin confer cancer stem cell (CSC) properties to mammary epithelial cells[12]. Snail expression is suppressed by glycogen synthase kinase-3β (GSK3β) at transcriptional and post-transcriptional levels[15,16], and the inhibition of GSK3β by small molecules induces EMT and promotes CSC phenotypes in breast cancer[17]. Despite the critical role of GSK3β in the regulation of CSCs phenotypes, the physiological and molecular mechanisms underlying its function remain unclear.

Breast CSCs exhibit a $CD44^{+}CD24^{-/low}$ antigenic phenotype with low expression of epithelial markers such as E-cadherin, and are characterized by high tumorigenicity and drug resistance[14,18]. Previously, we showed that *ribophorin II (RPN2) is a novel regulator of drug resistance in breast cancer and affects docetaxel resistance* by modulating the *N*-linked glycosylation of P-glycoprotein (ABCB1)[19]. To gain further insight into the regulation of mtp53 stability in CSCs, we screened for possible interactions between RPN2 and GSK3β. The present study suggests that the stabilization of mtp53 (R280K and del126-133) in breast cancer depends on RPN2 inhibition of GSK3β-mediated inactivation of HSP70 and HSP90 that are essential factors for the stabilization and oncogenic activities of mtp53.



Results

RPN2 is highly expressed in the CSC fraction. RPN2 was initially considered essential for the maintenance of CSCs because CSCs exhibit resistance to conventional chemotherapy[18,20]. To test this hypothesis, the expression of RPN2 in the CSC fraction was examined using two breast cancer cell lines, MCF7-ADR drug resistant human breast cancer cells and MDA-MB-231-D3H2-LN highly metastatic human breast cancer cells (MM231-LN). Since CSC fraction in breast cancer cells is reported to show the resistance to chemotherapy and the metastatic ability[21,22], we selected these two cell lines. Flow cytometry analysis showed that MCF7-ADR and MM231-LN cells comprise approximately 15% and 50% of CSCs, respectively (Fig. 1a). In breast CSCs, the CD44^{high}/CD24^{low} fraction shows higher tumorigenicity than the CD44^{high}/CD24^{high} fraction[23]. Flow cytometry and quantitative reverse transcription PCR (qRT-PCR) of MCF7-ADR and MM231-LN cells showed that RPN2 was more highly expressed in the CSC fraction than in the non-CSC fraction (Fig. 1b). In addition, the CSC fraction of MM231-LN showed high tumorigenicity in an animal model (Suppl. Fig. S1). Immunostaining analysis showed inverse correlation between RPN2 and E-cadherin expression in MM231-LN xenograft tumors (Fig. 1c).

To further investigate the role of RPN2 in CSCs, RPN2 knockdown experiments were performed in the two breast cancer cell lines, MCF7-ADR and MM231-LN, using lentivirus vectors expressing GFP and a small hairpin RNA against *RPN2* (shRPN2-site2) (Suppl. Fig. S2A and S2B). RPN2 knockdown reduced the E-cadherin negative fraction in MM231-LN cells as detected by flow cytometry analysis (Suppl. Fig. S3A). We also found that RPN2 knockdown induced Snail suppression in MM231-LN cells (Fig. 1d). Compared with MM231-LN shNC, a 40% decrease in *Snail* expression was observed in MM231-LN shRPN2 (Fig. 1d). Moreover, the GSK3β inhibitor CH99021 caused *Snail* upregulation in MM231-LN shRPN2 (Fig. 1d).

Consistent with the previous studies showing loss of epithelial phenotype by inactivation of p53[11,14], we confirmed that the ectopic expression of Snail and a point mutant p53 (R280K) in human mammary epithelial cells (HME cells) promoted the expression of Vimentin which is one of the mesenchymal cell markers (Fig. 2a, lane 3). Co-expression of Snail and C-terminal Myc-Flag tagged RPN2 (RPN2-MF) also promoted the expression of Vimentin in HME cells (Fig. 2a, lane 4). Next we established HME-Snail cell line that shows predominant mesenchymal phenotype and contains the CD44^{high}/CD24^{low} fraction[11] (Fig. 2b and 2d). Flow cytometry and western blot analysis revealed that while the expression of wtp53 suppressed Snail expression and reduced CSC fraction in HME-Snail cells, the expression of mtp53 or RPN2-MF did not alter the population of CSCs and Snail expression (Fig. 2b and 2d). More importantly, we also found that mtp53 (R280K) promoted the expression of RPN2 in HME-Snail cells (Fig. 2b lane 4). We also observed that mtp53 (R280K and R175H) promoted the protein stability of Snail and co-expression of RPN2-MF and mtp53 (R280K) induced the expression of N-cadherin in other human mammary epithelial cells (MCF10A cells) (Fig. 2c and Suppl. Fig. S3A). These results suggest that RPN2 plays an important role in the generation of CSC with EMT phenotype in breast cancer cells.

RPN2 regulates the tumorigenicity and metastasis of CSCs. The tumorigenicity of RPN2 knockdown cell lines was then examined using a 3D spheroid culture system[24]. In several cancer cell lines, CSCs form spheroids, which are essential for tumor onset in immunodeficient mice[14,24]. MM231-LN CSCs exhibited high tumorigenicity in an animal model (Suppl. Fig. S1). Compared with the control CSC fraction (MM231-LN shNC), the RPN2-knockdown CSC fraction (MM231-LN shRPN2) formed very few spheroids (Fig. 3a and b). To evaluate tumor formation by *in vivo* imaging, a limiting-dilution assay was performed using 6-week-old NOD/SCID mice that had been injected in the hind legs with 10^2 cells from a CSC fraction (CD44^{high}/CD24^{low}/GFP^{high}) derived from MM231-LN cells expressing firefly luciferase (Fig. 3c). The control CSC fraction formed tumors in all mice, whereas the RPN2-knockdown CSC fraction only formed one tumor from five injections (Fig. 3c and Suppl. Fig. S4A), and in that tumor, low RPN2 knockdown was confirmed by *in vivo* imaging (Suppl. Fig. S4B). Similar results were obtained with MCF7-ADR cells (Fig. 3c). The metastatic ability of RPN2 knockdown cell lines was examined next. In transwell migration assays, RPN2 knockdown reduced the invasiveness of CSCs in MM231-LN cells (Fig. 3d and e). After the transplantation of CSCs derived from MM231-LN shNC into the mammary fat pads of NOD/SCID mice, nodal and lung metastasis was observed in all mice (Fig. 3f). However, after transplantation of CSCs derived from MM231-LN shRPN2, nodal and lung metastasis was no longer observed (Fig. 3f and g), suggesting that RPN2 is essential for tumor formation and metastasis.

RPN2 antagonizes GSK3β function via physical interaction. Several studies show that GSK3β suppresses Snail expression at transcriptional and post-transcriptional levels[15,16], and the inhibition of GSK3β by small molecules induces EMT and promotes CSC phenotypes in breast cancer[17]. Our results also confirmed that the GSK3β inhibitor CH99021 suppressed E-cadherin expression in MCF7 cells (Suppl. Fig. S3B). In the present study, RPN2 knockdown reduced the E-cadherin negative fraction in MM231-LN cells via suppressing *Snail* expression (Fig. 1d and Suppl. Fig. S3C). These results indicate that RPN2 knockdown promotes GSK3β activation, and such activation is associated with the suppression of CSC phenotypes (Fig. 1 and 3).

The results above indicate that RPN2 regulates GSK3β activity in MM231-LN cells (Fig. 1c and d and Suppl. Fig. S3C); however, the *N*-linked glycosylation of GSK3β has not been previously observed. We therefore examined the physical interaction between RPN2 and GSK3β in MM231-LN cells by immunoprecipitation of endogenous RPN2 from MM231-LN cells. Since the phosphorylation of GSK3β at Tyr216 (GSK3β-216Y) is essential for the nuclear localization and activation of GSK3β[16], we examined the association of RPN2 with both Y^{216}-phosphorylated and unphosphorylated GSK3β (Fig. 4a). Immunoprecipitation analysis showed that RPN2 associated with both Y^{216}-phosphorylated and unphosphorylated GSK3β (Fig. 4a, lane 2). The physical interaction between RPN2 and GSK3β was also confirmed by using an anti-GSK3β antibody (Fig. 4b1, lane 2).

To determine the binding site of GSK3β on RPN2, in vitro binding assays were performed using extracts from 293T cells expressing RPN2-MF and C-terminal HA-tagged GSK3β (GSK3β-HA). Co-immunoprecipitation analysis revealed that GSK3β interacted with RPN2 in this extract (Fig. 4c and d, lane 2). Next, the binding region for GSK3β to RPN2 was examined using 293T cell extracts expressing RPN2-MF and GSK3β deletion mutants (Fig. 4e). Co-immunoprecipitation analysis revealed that the N-terminal amino acid region between 56–85 of GSK3β was critical for RPN2 binding (Fig. 4f, lane3).

To further investigate whether RPN2 antagonizes GSK3β activity via physical interaction, we examined the E-cadherin expression in 293T cells. In a control experiment, we confirmed that ectopic expression of N-terminal Flag-tagged Snail (Flag-Snail) induced E-cadherin suppression (Fig. 4g) and that expression of GSK3β-HA inhibited the Snail-mediated suppression of E-cadherin (Fig. 4g). Therefore, we examined whether ectopic expression of RPN2-MF with GSK3β-HA restores the Snail activity. Flow cytometry analysis showed that the expression of RPN2-MF restored the Snail-mediated suppression of E-cadherin (Fig. 4g). These results indicate that RPN2 inhibits the nuclear localization and activation of GSK3β via a physical interaction with its N-terminal region, and partially provide a

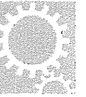


Figure 1 | RPN2 is essential for the maintenance of the CSC fraction. (a) The CSC fraction derived from MCF7-ADR and MM231-LN cells. (b) MCF7-ADR and MM231-LN cells were segregated by fluorescence-activated cell sorting (FACS) into $CD44^{high}/CD24^{low}$ and $CD44^{high}/CD24^{high}$ subsets; sorted subsets were then compared for RPN2 expression by quantitative real-time PCR (qRT-PCR). Each data point is the average of three experiments. (c) Immunohistochemistry (IHC) for RPN2 (Top panel) and E-cadherin (Bottom panel) in MM231-LN tumors. MM231-LN xenografts were grown for 5–6 weeks after fat pad injection. Sections are representative of at least four mice analyzed per group. Scale bar, 200 μm. (d) RPN2 knockdown suppressed *Snail* expression via GSK3β activation. In MM231-LN cells, RPN2 and Snail mRNA expression was monitored by qRT-PCR after 72 h of treatment with a GSK3β inhibitor (CHIR99021, 3 μM). (n = 3, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).