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	Bone marrow derived DCs	DC2.4 (mouse dentritic cell line)		[a]	[44]
miR-148a					
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].

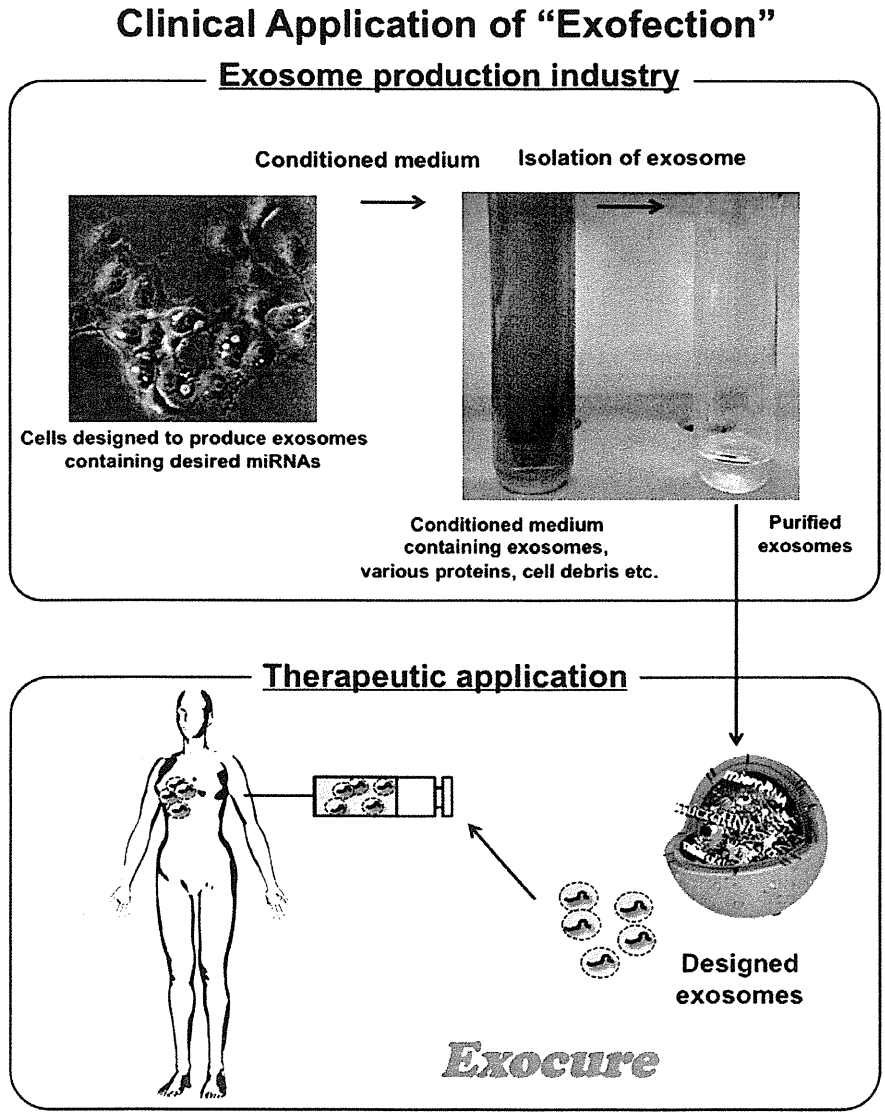

Fig. 1. A schematic explanation of "exocure". Exosome containing desired miRNAs, such as miR-16, miR-143 and so on, is produced by the "exosome production industry", and then these designed exosomes are delivered to cure the patient's disease. For this purpose, some of the issues which are showed in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

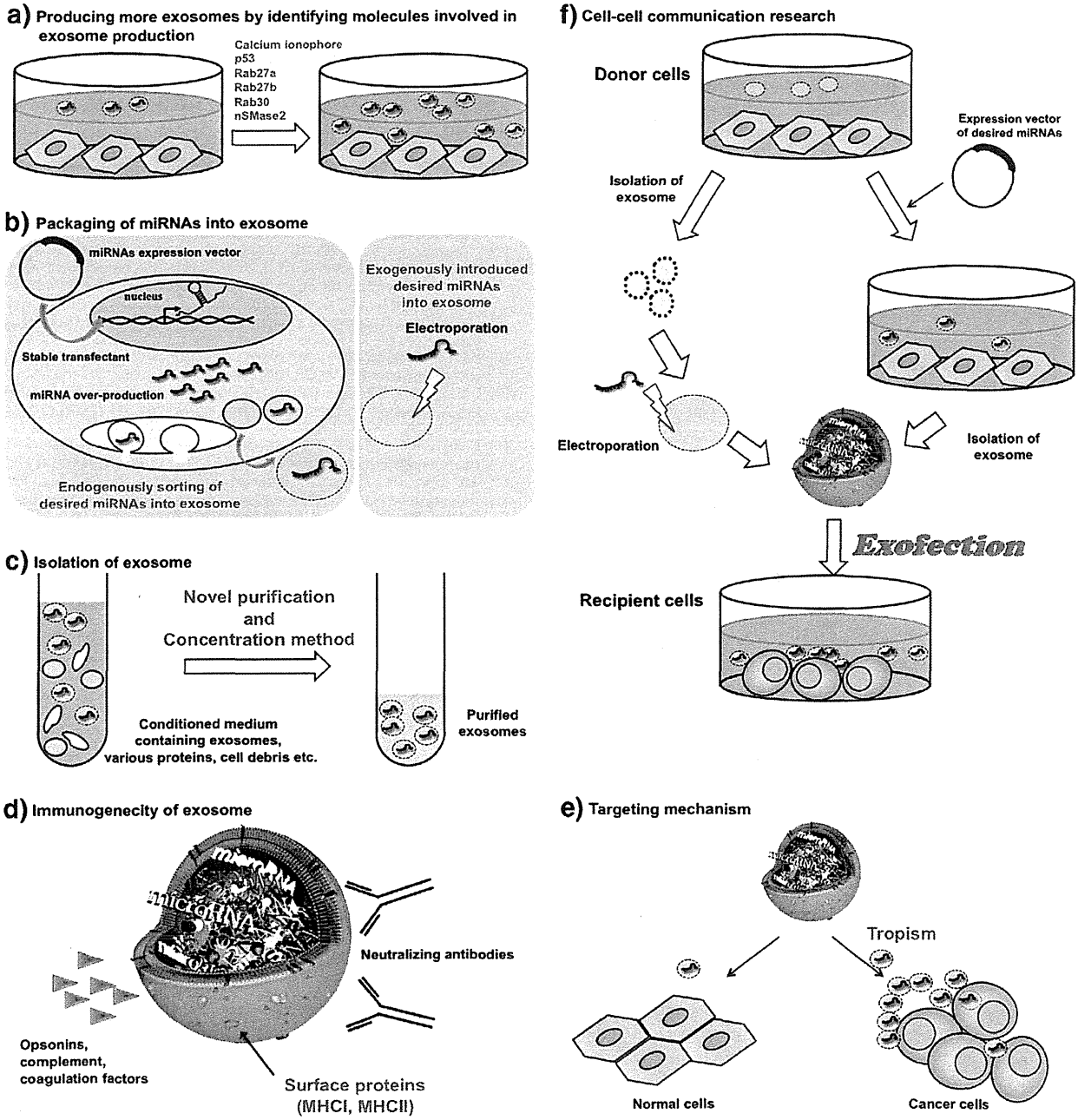

Fig. 2. Issues for the clinical use of "exocure". A schematic explanation of how the novel treatment "exofection" was established and the issues to be solved. To prepare exosomes containing tumor-suppressive miRNAs suitable for clinical usage, clarifying the mechanisms of exosome secretion is important. 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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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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Review Article

RNAi Therapeutics and Applications of MicroRNAs in Cancer Treatment

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RNA interference-based therapies are proving to be powerful tools for combating various diseases, including cancer. Scientists are researching the development of safe and efficient systems for the delivery of small RNA molecules, which are extremely fragile in serum, to target organs and cells in the human body. A dozen pre-clinical and clinical trials have been under way over the past few years involving biodegradable nanoparticles, lipids, chemical modification and conjugation. On the other hand, microRNAs, which control the balance of cellular biological processes, have been studied as attractive therapeutic targets in cancer treatment. In this review, we provide an overview of RNA interference-based therapeutics in clinical trials and discuss the latest technology for the systemic delivery of nucleic acid drugs. Furthermore, we focus on dysregulated microRNAs in human cancer, which have progressed in pre-clinical trials as therapeutic targets, and describe a wide range of strategies to control the expression levels of endogenous microRNAs. Further development of RNA interference technologies and progression of clinical trials will contribute to the achievement of practical applications of nucleic acid drugs.

Key words: RNA interference – microRNA – DDS – Cancer

INTRODUCTION

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants initiated by double-stranded RNA (dsRNA). It is the most significant recent contribution to the field of cell biology, and Fire and Mello who discovered it were awarded the Nobel Prize for Medicine in 2006 (1). The silencing technology to suppress the expression of pathologically or physiologically important genes by using small interfering RNA (siRNA) is applicable to many kinds of research or therapeutics for human diseases caused by specific genes, which are difficult to regulate through traditional approaches. Indeed, as the initial description of RNAi in animals, the development of RNAi-based therapies has provided a powerful new arsenal against various human diseases, such as age-related macular degeneration (AMD) (2,3), respiratory syncytial virus (RSV) infection (4), neurodegenerative disorders (5) and cancers (6–8).

On the other hand, in recent years, microRNAs (miRNAs) have been studied as regulators of gene expression in crucial biological processes, including cell development, differentiation, apoptosis and proliferation (9,10). miRNAs are non-coding small RNAs (~22 nt) which are processed from endogenously expressed transcripts and induce translational suppression and mRNA degradation in animals, plants and viruses (11,12). miRNAs are first transcribed as primary miRNA (pri-miRNA) transcripts by RNA polymerase II and then processed by Drosha in the nucleus to generate

~60–100 nt precursor-miRNA (pre-miRNA) with a hairpin-like structure. After pre-miRNAs are transported to the cytoplasm by Exportin-5, they are processed into mature miRNA duplexes by Dicer assembled with transactivating response RNA-binding protein and protein activator of PKR (13,14). Finally, one strand of the mature miRNA duplex, a guide strand, is incorporated into the Argonaut-containing RNA-induced silencing complex, which induces either cleavage or translational repression of targeted mRNAs based on their sequences (Fig. 1). Once the miRNAs are unbalanced or the functions are disordered, they can be involved in the initiation and development of fatal human ailments, including cancer (15). Indeed, many reports have shown that the widespread disruption of miRNAs was correlated with the initiation and progression of human cancer and demonstrated that an injection with synthetic RNA molecules mimics tumor suppressor miRNAs or the inhibitors of oncogenic miRNA (onco-miR) can switch dozens of cancer-related signals on or off (16). In other words, miRNAs are potential therapeutic tools for cancer treatment, representing a superior molecular target approach to the traditional low-molecular compound approach. However, for the realization of RNAi-based therapies using siRNAs, synthetic miRNAs and miRNA inhibitors, more continuous improvements will be required. For example, the technology to avoid unwanted innate immune responses, instability of nucleic acid *in vivo* and off-target side effects strikingly decreases the levels of potency and efficacy of RNAi effector molecules (17–19). Thus, the development of drug delivery systems (DDS) for RNAi therapeutic strategies that are safer, more stable and more effective is a paramount consideration.

Although clinical applications of RNAi-based therapies have not been fully realized, numerous pre-clinical studies in animal models of human disease are providing opportunities for practical use. In this review, we provide an overview of the current clinical and pre-clinical trials of RNAi therapies and discuss strategies toward a pathway of miRNA to practical applications for cancer therapy from the viewpoint of RNAi DDS.

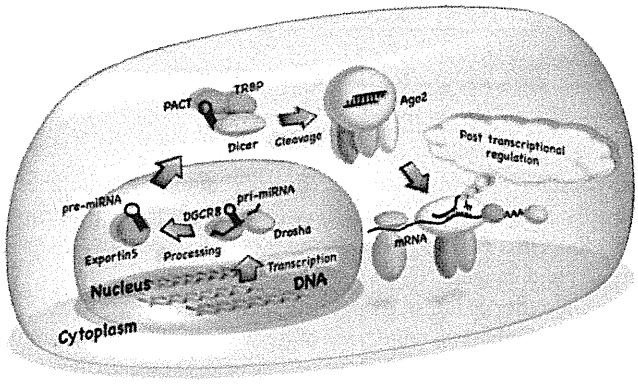

Figure 1. Cellular mechanisms of RNA interference pathway in mammals. First, primary miRNAs (pri-miRNAs) are transcribed by RNA polymerase II and are cleaved by the enzyme Drosha into ~70 nucleotides as precursor-miRNAs (pre-miRNAs). Next, these pre-miRNAs are exported to the cytoplasm with Exportin-5 and are cleaved to double-strand RNAs that do not contain a loop by Dicer. These duplexes are then associated with Argonotc2 (Ago2), and one strand is removed. This RNAi-induced silencing complex (RISC) containing the guide strand triggers post-transcriptional regulation of target mRNA depending on the seed sequence of miRNAs.

RNAi THERAPEUTICS DEVELOPMENT PIPELINE

In the development of RNAi technology for therapeutic medication, since the first demonstration of RNAi triggered by siRNA in mammalian cells in 2001 (20,21), some risk-taking biotechnology companies, such as Sirna Therapeutics, Silence Therapeutics and Tekmira, started to establish a platform to develop the new technology using primarily siRNA. At first, some pharmaceutical companies ascribed the RNAi to research for directed gene silencing; however, after the first exploration of *in vivo* gene knockdown (22), major pharmaceutical firms, such as Medtronic, Novartis and Merck, became involved in clinical applications. Observers were surprised by the acquisition by Merck and Roche of Sirna Therapeutics for more than one billion USD. RNAi was considered an exceptional technology for the knockdown of therapeutic target genes, and scientists anticipated that it would significantly shorten the drug development timeline. However, as a consequence of the global economic turmoil that began in 2008 and the slump in development of DDS for RNAi medicine, companies such as Merck, Pfizer, Abbott Labs and Roche were forced to curtail research in these fields. In particular, the fact that Roche halted its development of RNAi technology in 2010 was a shock throughout the industry. The Roche decision resulted in a loss of confidence in the company's ability to innovate, and their withdrawal from RNAi research was followed by other companies. However, the clinical pipeline of RNAi therapies using siRNA has been gradually growing since approximately 2011 as the RNAi technology has matured.

As shown in Table 1, there are many candidates for clinical development in 2012. In particular, there are a number of sites for topical or local administration, such as the skin, retina and airways, which permit safe and efficient delivery without unwanted side effects. For example, according to some recent animal experiments, transtympanic administration of siRNA targeting NOX3 is significantly useful for the attenuation of cisplatin ototoxicity (23). Furthermore, Paller *et al.* (24) at Northwestern University showed that spherical nucleic acid nanoparticle conjugates gold cores surrounded by immobilized siRNA directed against EGFR can be topically delivered more stably into mouse and human skin without undesirable toxicity. Thus, accessibility is a key requirement for successful RNAi *in vivo* to be delivered tissue or cell specifically. Since around 2008, however, the development pipeline has shifted from local to systemic delivery because more advanced delivery vehicles for systemic

Table 1. Current states of clinical candidate pipeline for RNAi therapy

ClinicalTrials.gov identifier	Drug	Route	Delivery	Disease	Target	Phase	States	Company
NCT00499590	Bevasiranib	IVT	Naked siRNA	Wet AMD	VEGF	III	Terminated	Opko Health
NCT00363714, NCT00395057	AGN211745/ Sirna–027	IVT	Naked siRNA	AMD	VEGF-R1	II	Terminated	Allergan/ Sirna
NCT01065935, NCT00658086	ALN-RSV01	Nebulization	Naked siRNA	RSV infection after lung transplantation	RSV Nucleocapsid	II	Completed	Alnylam
NCT00306904	Bevasiranib	IVT	Naked siRNA	DME	VEGF	II	Completed	Opko Health, Inc.
NCT01445899	PF-04523655	IVT	Naked siRNA	DME	RTP801	II	Recruiting	Quark Pharma
NCT01200420	miravirsen	SC	Naked LNA	HCV	miR-122	II	Completed	Santaris Pharma
NCT01551745 NCT01505166	FANG vaccine	*Ex vivo*, Intradermal	Electroporation	Ovarian cancer, colon cancer	Bi-shRNA-Furin and GM-CSF	II, II	Recruiting, Recruiting	Gradalis, Inc.
NCT00802347	I5NP	IV	Naked siRNA	DGF in kidney transplantation	P53	I/II	Recruiting	Quark Pharma
NCT01227291	SYL040012	Ophthalmic drops	Naked siRNA in ophthalmic drops	Glaucoma and ocular hypertension	Adrenergic receptor beta-2 siRNA	I/II	Completed	Sylentis
NCT00725686, NCT00713518	PF-04523655	IVT	Naked siRNA	Wet AMD	RTP801	I, II	Completed	Pfizer/Quark
NCT00716014	TD101	Intralesional	Naked siRNA	Pachyonychia congenita	Keratin 6a N171K mutant mRNA	Ib	Completed	TransDerm/ IPCC
NCT00882180, NCT01158079	ALN-VSP02	IV	SNALP	Liver cancer, solid tumors	KSP and VEGF	I, I	Completed	Alnylam
NCT00554359, NCT00683553	I5NP	IV	Naked siRNA	AKI for major cardiovascular surgery	P53	I, I	Completed, terminated	Quark Pharma
NCT01148953	ALN-TTR01	IV	SNALP	TTR-mediated amyloidosis	Transthyretin	I	Completed	Alnylam
NCT00689065	CALAA-01	IV	RONDEL	Solid cancer	RRM2	I	Active	Calando Pharma
NCT00466583	EZN-2968	IV	Naked LNA	Advanced solid tumor, lymphoma	HIF-1a	I	Completed	Santaris Pharma
NCT01120288	EZN-2968	IV	Naked LNA	Liver metastases	HIF-1a	I	Recruiting	NCI
NCT00672542	siRNA in dendritic cells	*Ex vivo*, Intradermal	Electroporation	Metastatic melanoma	Immunoproteasome subunits LMP2, LMP7, MECL1	I	Active	Duke University
NCT01061840	FANG vaccine	*Ex vivo*, Intradermal	Electroporation	Solid tumors	Bi-shRNA-Furin and GM-CSF	I	Recruiting	Gradalis, Inc.
NCT01064505	QPI-1007	IVT	Naked siRNA	Optic atrophy	Caspase-2	I	Active	Quark Pharma
NCT00938574	Atu027	IV	AtuPLEX	Advanced solid cancer	PKN3	I	Completed	Silence Therapeutics
NCT01188785	siG12D LODER	EUS biopsy needle	LODER polymer	Pancreatic ductal adenocarcinoma	KRASG12D	I	Recruiting	Silenseed Ltd
NCT01262235	TKM-080301	IV	SNALP	Cancer	PLK1	I	Recruiting	Tekmira
NCT00927459	PRO-040201	IV	SNALP	Hypercholesterolemia	Apo B	I	Terminated	Tekmira

AKI, acute kidney injury; AMD, age-related macular edema; DGF, delayed graft function; DME, diabetic molecular edema; HCV, Hepatitis C Virus; IV, intravenous; IVT, intravitreal; KSP, Kinesin Spindle Protein; LNA, locked nucleic acids; NCI, National Cancer Institute; PEG, polyethylene glycol; PLK1, Polo-like Kinase I; RRM2, Ribonucleotide Reductase M2; RSV, respiratory syncytial virus; SNALP, stable nucleic acid lipid particle; TF, transferrin; TTR, transthyretin; VEGF, vascular endothelial growth factor; SC, subcutaneous.
*From ClinicalTrials.gov.

application, such as stable nucleic acid lipid particles (SNALPs) and RNAi/oligonucleotide nanoparticle delivery (RONDEL), are available. These technologies have been shown to be effective *in vivo* (25–27), and progress is being achieved in some clinical trials (ALN-VSP02, ALN-TTR01, CALAA-01, TKM-080301, PRO-040201). In cancer treatment, siRNAs targeting polo-like kinase I (PLK1), kinesin spindle protein (KSP) and vascular endothelial growth factor, which are formulated with SNALP or RONDEL, have been developed as candidate pipelines in Phase I (Table 1).

DRUG DELIVERY SYSTEM FOR SYNTHETIC OLIGONUCLEOTIDE

Nucleic acid medicines, including siRNA, miRNA and anti-miRNA, work only after they penetrate hydrophobic cellular membranes. However, it is not easy for them to go through the lipid bilayer membrane without their carrier because synthetic oligonucleotides are negatively charged. In addition to this, RNAs are very easily degraded by RNase *in vivo*. Accordingly, assisting carriers or chemical modifications for the progression of the transmembrane transport and for the inhibition of the degradation by serum RNases are required. Historically, viral and non-viral delivery has been utilized (Table 2). In a viral delivery system, it was reported that an adenovirus carried short hairpin RNA (shRNA) expression vector targeting angiotensin type 1 (AT1) delivered into the brain intracerebroventricularly (ICV) (28) and that the miR-23b expression vector and miR-23b sponge worked in inflammatory autoimmune diseases *via* intra-articular (IA) infection (29). An adeno-associated virus (AAV) was also successful at carrying a miRNA cluster into the muscle and shRNA vectors targeting mutant huntingtin into the brain by topical administration (30,31). Furthermore, miR-34a treatment prevented lung cancer initiation and progression *via* transtracheal infection, and shRNA targeting superoxide dismutase 1 (SOD1) inhibited amyotrophic lateral sclerosis progression by lentiviral-mediated RNAi (32,33). The herpes simplex virus, which commonly causes an eruption of fluid-containing vesicles on the mouth, lips or face, also has potential for cancer treatment and therapeutic pain relief (34,35). Thus, viral-mediated gene silencing is very useful for local infection, particularly at sites that make frequent administration difficult. Although viral delivery has frequently shown higher efficiency than that by non-viral systems, preliminary clinical studies have shown that it triggered strong inflammatory reactions (36), and these delivery vectors have caused the death of several patients in the clinic (37,38). Thus, understanding the details of the inflammatory mechanism and developing safer viral vehicles are important tasks ahead.

On the other hand, the focus has recently been on the non-viral approach because of its advantages over viral vectors, such as non-immunogenicity, low production cost and easy quality control. This approach requires an optimized delivery reagent, such as a cationic lipid, polymers, nanoparticles, carbon nanotubes and atelocollagen (Table 2). In cancer treatment, atelocollagen or cationic liposome- or polymer-mediated transfection reagents have commonly been used to deliver siRNA or miRNA to cells *in vitro* and *in vivo*. In particular, a number of reports have demonstrated a significant anti-cancer effect caused by systemic delivery of siRNA with cationic liposome (39–41). Similarly, a cationic polymer, polyethyleneimine, was commercialized as *in vivo*-jetPEI™, provided by Life Technologies, and was used to successfully deliver siRNAs to cancer cells in animals (42,43). In addition, atelocollagen can be obtained from type I collagen of calf dermis and has also been expected to be a useful carrier because of its low immunogenicity and efficiency (8,44–46). In case of miRNA therapy, a tumor-suppressive miR-16 mimic was successfully delivered by the systemic approach using atelocollagen, and it dramatically inhibited the growth of metastatic prostate cancer (47). Furthermore, chemically functionalized carbon nanotubes also show potential for novel biological applications for the delivery of Caspase-3 siRNA into the brain by topical injection into the cerebral cortex and reduced neurodegeneration without toxic side effects (48).

In a recent study, the focus was on highly stabilized nanoparticles, and these nanoparticles made the systemic delivery system dramatically more efficient (25,49–52). For example, synthetic miR-34a mimic, which was incorporated into cholesterol, and the cationic liposome *N*-[1-(2,3-dioleoyloxy)]-*N*,*N*,*N*-trimethyl ammonium propane (DOTAP) (1:1 mol/mol) and polyethylene glycol (PEG)-conjugated CG4-targeting single-chain antibody fragment were efficiently delivered into melanoma and inhibited lung metastasis (53). The nanocarrier 'SNALP' by Tekmira pharmaceuticals is one of the technologies with the most potential in the clinical pipeline. SNALP is a PEG-grafted monolamellar liposome that can easily avoid opsonization and subsequent recognition by the macrophages because the hydrophilic nature of PEG constructs an aqueous coating on its particle surface (54). In the work of Judge *et al.*, SNALP-formulated siRNAs against PLK1 and KSP displayed significant anti-tumor effects in liver tumor model mice (26). Successful results have already been reported in the treatment of transthyretin-mediated amyloidosis, hypercholesterolemia, Ebola virus infection (49) and cancer (50). The clinical trials have been identified as NCT00882180, NCT01158079, NCT01148953, NCT01262235 and NCT00927459 in the ClinicalTrials.gov database (http://clinicaltrials.gov).

The effective systemic delivery of siRNA or miRNA toward target cells or tissues has been enormous challenge for RNAi therapy. Indeed, naked siRNAs are rapidly eliminated by the kidneys, and nanoparticle-formulated siRNAs have a tendency to accumulate in the liver. In particular, their suitability for cancer cells depends on the enhanced permeability and retention effect of nanoparticles. To solve these problems, combined use with orienting molecules, such as a cell-specific ligand, can increase the cell or tumor

Table 2. Technologies for drug delivery systems in RNAi therapy

Delivery	Tissue	Route	RNA	References
Viral vector				
Adenovirus	Articulation	IA	miR-23b	(29)
	Brain	ICV	AT1a, AT1b shRNA	(28)
Adeno-associated virus	Muscles	IM	Anti-VEGF miRNA cluster	(30)
	Brain	Intrastriatal	mHTT shRNA	(31)
Lentivirus	Lung	Transtracheal	miR-34a	(32)
	Spinal cord	Intraspinal	SOD1 shRNA	(33)
Herpes simplex virus	Dorsal root ganglia	Injection into the sciatic nerve	Trpv1 shRNA	(34)
	Glioma	IT	EGFR shRNA	(35)
Non-viral reagent				
Liposome				
Oligofectamine	Colon cancer	IP/IV	B-catenin siRNA	(40)
DOTAP	Liver, spleen	IV	GFP siRNA	(39)
LIC-101	Liver metastasis	IV/SC	BCL-2 siRNA	(41)
PEI	Ovarian canner	IP/SC	HER-2 siRNA	(42)
	Glioblastoma	IP/SC	PTN siRNA	(43)
Nanoparticle				
SNALP	Ebola virus	IP/SC	ZEBOV siRNA	(49)
	Lung cancer	IV	miR-34a/let-7	(50)
RONDEL	Melanoma	IV	RRM2 siRNA	(25)
	Ewing's sarcoma	IV	EWS-FLI 1 siRNA	(51)
AtuPLEX	Prostate/pancreatic cancer	IV	PKN3 siRNA	(52)
DOTAP, cholesterol and PEG	Melanoma	IV	c-Myc/MDM2/VEGF siRNA and miR-34a	(53)
Atelocollagen	Testicular cancer	IT	HST-1/FGF-4 siRNA	(45)
	Osteosarcoma	IV	miR-143	(112)
	Prostate cancer		miR-16	(47)
HDI	Liver	IV	HBV siRNA	(113)
Carbon nanotube	Brain	Into the cerebral cortex	Caspase-3	(48)

ApoB, Apolipoprotein B; AT1, Angiotensin type 1; DDAB, dimethyldioctadecylammonium bromide; DOTAP, (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium methylsulfate; HBV, Hepatitis B Virus; HDI, hydrodynamic tail vein injection; HER-2, human epidermal growth factor receptor 2; IA, intra-articular, ICV, intracerebroventricular; IM, intramuscular; IP, intraperitoneal; IT, intratumor; IV, intravenous; mHTT, mutant huntingtin; PBAVE, poly butyl and amino vinyl ethers; PEI, polyethyleneimine; PKN3, Protein Kinase N3; PPARA, peroxisome proliferator-activated receptor alpha; PTN, pleiotrophin; SC, subcutaneously; SLN, solid lipid nanoparticle; SOD1, superoxide dismutase 1; ZEBOV, The Polymerase (L) Gene of the Zaire Species of Ebola Virus.

specificity and delivery efficiency (55–57). Calando's cyclodextrinpolymer-based delivery platform (RONDEL) consists of cyclodextrin-containing polycation, and adamantine-coupled PEG-stabilized some ligands, such as transferrin (TF), and siRNA or miRNA (Fig. 2). This siRNA delivery platform was conceived by Hu-Lieskovan *et al.* in 2005 (51). The TF receptors are known to be upregulated in malignant cells, and TF-stabilized particles are taken up into cancer cells by TF receptor-mediated endocytosis and subsequent release into the cytoplasm in a pH-dependent manner (25). Phase 1b clinical trials of CALAA-01, including the M2 subunit of ribonucleotide reductase (RRM2) targeting siRNA, are being conducted as a novel RNAi therapy for multiple types of solid tumors.

CHEMICAL MODIFICATIONS FOR OLIGONCLEOTIDES

In addition to the nanocarriers mentioned above, others are being sought through chemical modifications. The purpose of such modifications can be permeability into the cells, specificity for specific tissues and stability against nuclease degradation (Fig. 3 and Table 3). For example, as a

permeability-enhancing factor, the covalent conjugation of the lipophilic molecule assists siRNA or miRNA to penetrate into the cellular cytoplasm and trigger gene silencing *in vivo* (58). In particular, high-density lipoprotein (HDL)-conjugated siRNAs are selectivity taken up by the gut, kidney and steroidogenic organs *via* the HDL receptor, scavenger receptor class B, type I (SR-BI) (59–62). In contrast, low-density lipoprotein (LDL)-conjugated siRNAs are efficiently internalized into the hepatocytes after binding to the LDL receptor (59). The Arrowhead Research Corporation demonstrated that the co-injection of cholesterol-siRNA and hepatocyte-targeted endosomolytic polymer achieved high-level target gene knockdown with low doses of cholesterol-siRNA in non-human primates (63). The company is using this strategy and a polymer-based siRNA delivery platform named dynamic polyconjugate polymer in ARC-520, which is a hepatitis B clinical candidate.

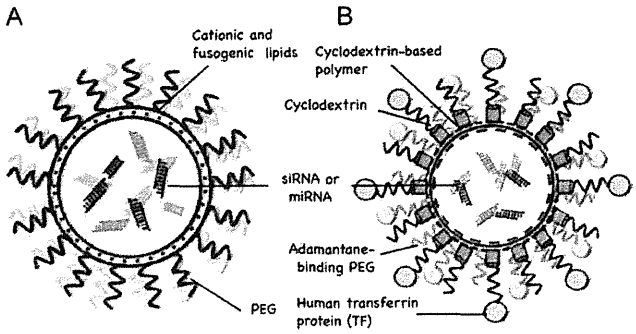

Figure 2. Delivery technology for RNAi therapy. (A) Stable nucleic acid lipid particle (SNALP). The bilayer consists of cationic and neutral lipids and is coated by PEG. The diameter is ~100 nm. (B) RNAi/oligonucleotide nanoparticle delivery (RONDEL). RNAs are protected from degradation in serum by the cyclodextrin-conjugated polymer. The complexes are <100 nm in diameter. In aqueous solution, adamantane easily binds to cyclodextrin as a result of hydrophobic attraction.

In another example, nanoparticles composed of poly (lactic-*co*-glycolic acid) were modified with a cell-penetrating peptide, penetratin, and used for the systemic delivery of the miR-155 inhibitor in the mouse model of lymphoma (64).

On the other hand, cell-specific factors, such as aptamers (65,66), peptide (64,67), antibodies (68,69) and agonists (56), can enhance cell specificity in cases of systemic administration into experimental animals. For example, octaarginine-modified liposomal particles were used to suppress an endogenous gene in the liver at low concentrations of siRNA without any toxicity (67). Usually, targeting proteins were conjugated to cationic bridges, such as polylysine or protamine, which can mediate uptake of nucleic acids, to link targeting proteins to effector oligonucleotide (68,70–72). In contrast, the siRNA-aptamer chimeras have also been of interest because a completely RNA-based approach may have important advantages over other methods for targeted delivery of siRNAs in terms of cost, productivity, safety and flexibility regarding chemical modification. RNA aptamers are single-stranded oligonucleotides and bind with high affinity to specific molecular targets, such as small molecules, proteins and nucleic acids, with their 3D structure (65,66). Here, although antibody-mediated siRNA delivery is required for the biological production of antibodies and antibody-siRNA conjugations by using a linker such as PEG, chimeric aptamer-siRNA can be synthesized as a single unit at once. However, for the utilization of chimeric aptamer-siRNA, more structured RNAs capable of binding with higher affinity and specificity have been required.

Stabilization in serum has been developed for the inhibition of the nuclease activity. Indeed, the backbone linkage introduced phosphorothioate (PS) or the sugar conjugated with protecting groups such as 2′-*O*-methyl (2′-*O*-Me), 2′-fluoro (2′-F), 2′-*O*-(2-methoxyethil) (2′-*O*-MOE), 5′-methylenephosphonate (5′-MP) and 5′-(E)-vinyl-phosphonate (5′-VP)

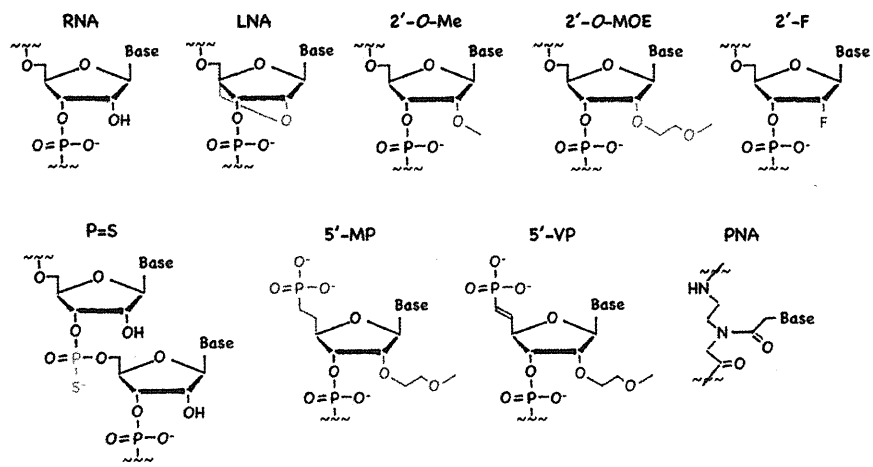

Figure 3. Chemical modifications for stability. Sugar, backbone and base modifications are illustrated. Shown are locked nucleic acid (LNA), phosphorothioate (P = S), 2′-*O*-methyl (2′-*O*-Me), 2′-fluoro (2′-F), 2′-*O*-(2-methoxyethil) (2′-*O*-MOE), 5′-methylenephosphonate (5′-MP), 5′-(*E*)-vinyl-phosphonate (5′-VP) and peptide nucleic acid (PNA).

Table 3. Chemical modifications for permeability and specificity

Chemical modification	Tissue	Factor	Route	RNA	References
PEG, PBAVE and ligand	Liver	NAG	IV	ApoB and PPARA siRNA	(114)
Aptamer	PSMA-positive prostate cancer	Anti-PSMA aptamer	IT	PLK-1/BCL-2 siRNA	(65)
	HIV-infected T cells	Anti-gp120 aptamer	IV	tat/rev siRNA	(66)
Cholesterol	Colon adenocarcinoma	Cholesteryl oligo-D-arginine	IT	VEGF siRNA	(58)
	Liver	HDL/LDL	IV	ApoB siRNA	(59)
Antibody	HIV-infected T cells	Anti-HIV Envelope Fab	IV	gag siRNA	(68)
	Hepatocellular carcinoma	Anti-EGFR Fab	IV	Luciferase siRNA	(69)
Peptide	Liver	Octaarginine	IV	SR-B 1 siRNA	(67)
	Lymphoma	Penetratin	IV/IT	Anti-miR-155-PNA	(64)
Agonist	TLR9 + myeloid cells and B cells	Anti-TLR9 agonist	IV/IT	Stat3	(56)

NAG, *N*-acetylgalactosamine; TLR, toll-like receptor.

enhance the resistance against exonuclease or endonuclease activity (73,74) (Fig. 3). Currently, the most consequential modification is the PS inter-nucleotide linkages that have been developed in the history of anti-sense oligonucleotides and have contributed to remarkable stabilization of double-strand RNA as well as the single-strand oligonucleotide (75,76). However, the influence of chirality in the phosphorus atom on the stability and the activity of duplexes is not entirely understood. Therefore, further investigation of the thermodynamic features and physiological activity with regard to the assignment of the absolute configuration will be required for therapeutic applications.

As reported above, a number of chemical modifications have been produced, which have enhanced the potential of siRNA, miRNA, miRNA inhibitors and anti-sense oligonucleotides. However, it has been required that the optimization of the modifications need to be optimized, as their efficiency depends on the position and combination. In 2012, chemical modifications were optimized for single-stranded siRNAs (ss-siRNAs), and the change was an important advancement for the practical application of RNAi therapeutics. It was shown that ss-siRNA with a number of chemical modifications, such as 5′-phosphonate and 2′-MOE-modified 5′-terminal nucleotide, 2′-F and 2′-*O*-Me motifs with contiguous PS modifications and 2′-MOE-modified adenosine dinucleotide at the 3′ terminus and C16 modification, brought about significant and efficient target gene silencing *in vivo via* the Ago2-mediated RNAi pathway (74). Furthermore, chemically modified ss-siRNAs targeting mutant huntingtin mRNAs have been employed as a novel nucleic acid drug for therapeutic application for Huntington's disease (77). Although single-stranded RNAs (ssRNAs) have been shown to have extremely rapid degradation in serum and poor activities so far (78,79), they have advantages, such as the absence of risk of undesirable off-target effects by passenger strand and the potential of systemic delivery without complex lipid formulations that sometimes trigger the inflammatory toxicities (80). Hence, these stabilized ssRNAs are expected to place RNAi therapy in a prominent class of nucleic acid drugs.

DYSREGULATED MIRNA AS THERAPEUTIC TARGET IN CANCER TREATMENT

The alterations of miRNA expression profiling are significantly related with cancer initiation and progression. To identify dysregulated miRNAs in the physiological and pathological pathway of cancer malignancy is the first step for therapeutic applications. Generally, the widespread disruption of miRNAs is caused by at least three different mechanisms: the loss, amplification or mutation of a fragile cancer-related genomic region; the change of epigenetic control; and the abnormality of miRNA-processing steps. The genetic change has the potential to affect radically the abundance of miRNA, and it was reported that >50% of miRNAs locate on the fragile genomic region in cancer (81–83). For instance, a significant downregulation of miR-15 and miR-16, which is caused by deletion or mutation in chromosome 13q14.3, was observed in 70% of patients with chronic lymphocytic leukemia.

On the other hand, CpG-island hypermethylation and histone modification as good markers for functional miRNA have also been investigated by using 5-aza-2′-deoxycytidine and a histone decetylase inhibitor, such as 4-phenylbutyric acid or trichostatin A (84–86). For example, miR-124a that regulates the expression of cyclin D kinase 6 was located in three chromosome loci, 8p23.1, 8q12.3 and 20q13.33, and these regions were hypermethylated in 75% of patients with primary colorectal tumors (87). In addition to genetic and epigenetic validation, alterations of the protein machinery related to the biogenesis of miRNA might impair global