Versatile Applications of microRNA in Anti-Cancer Drug Discovery: From Therapeutics to Biomarkers

Haruhisa Iguchi, Nobuyoshi Kosaka and Takahiro Ochiya*

Section for Studies on Metastasis, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

Abstract: Over the past several years, microRNAs (miRNAs) have been identified as a fine-tuner in a wide array of biological processes, including development, cell growth and metabolism. Recent studies have shown that many kinds of miRNAs act as oncomirs or tumor suppressors in tumors where the miRNA genes are up- or down- regulated, respectively. These dysregulations occur through a variety of mechanisms, such as genetic alterations, epigenetic repression or altered expression of transcription factors which target miRNAs. The aberrant expressions of miRNAs are observed not only in tumor lesions but also in plasma and serum of cancer patients. These characteristics of miRNAs have created extensive interest in tapping into them for diagnosis and prognosis as well as drug discovery in cancer therapy.

In this literature, the significance of miRNAs in tumor initiation and development is first reviewed. Second topic is extracellular miRNAs as biomarkers for cancer classification and prediction. Further, we focus on secretory machinery of miRNAs and share new evidence suggesting that extracellular miRNAs can play biological roles beyond mere biomarkers. Extending this concept, our hypothetical model that extracellular miRNAs may function as a signaling molecule in a crosstalk between cancer cells and their surrounding cells is presented. Finally, we discuss the potential of miRNAs for therapeutic applications in clinical oncology.

Keywords: microRNA, cancer, dysregulated expression, biomarker, secretion, microRNA based drug.

INTRODUCTION

Since microRNAs (miRNAs), small 20-22 nucleotide-long members of the non-protein-coding RNA family, were discovered in *Caenorhabditis elegans* in 1993, they have been identified in the vast majority of living organisms, including yeasts, plants, and animals [1, 2]. Through the exquisite processing machinery, miRNAs become small but powerful bioactive molecules. One strand (guide strand) of the mature duplex miRNAs interacting with Argonaute proteins are selectively incorporated into an RNA-induced silencing complex (RISC), in which the miRNAs suppress translation of their target genes through binding to partially complementary sites, usually located in the 3' untranslated regions (3'-UTR) of the target mRNAs [3]. The ability to interact with imperfectly matched sequences allows miRNAs to impact expressions of multiple genes and subsequently play a crucial role in a variety of biological processes [4-6].

Despite the rapid and dramatic progress on the basic study of miRNA maturation process, the perspective of miRNA biology is not fully uncovered. In a sharp contrast with the guide strand of miRNAs, the physiological significance of the opposite strand (passenger strand) remains to be obscure. However, several research groups have recently lent evidence that the passenger strands can also function as a translational inhibitor [7-10]. Furthermore, mounting evidence suggests that miRNAs are packaged in exosome fractions, followed by release from producing cells into the circulation in the body fluids of whole body [11-13]. Exosomes are small membrane-bound vesicles (50 to 100 nm in diameter) of multivesicular bodies (MVB) released on exocytic fusion of this organelle with plasma membranes [14]. A variety of cells, from yeasts to mammalian cells, use MVB as a means for sorting proteins to recycling or degradation. The proteins under this selection process are modified with ubiquitin molecules, which are recognized by endosomal sorting complex required for transport (ESCRT) system on MVB [15]. ESCRT machinery comprising several protein complexes, such as ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III, functions in the biogenesis of MVB, cytokinesis, and the budding of enveloped RNA viruses. More intriguingly, high levels of exosomes were observed in plasma of melanoma patients, suggesting the link between cancers and secretory exosomal miRNAs [16].

Recent studies have defined that many different kinds of miRNAs contribute to cancer pathogenesis. The expression profiles indicated that aberrant expressions of miRNAs are implicated in cancer development and progression. Reduced and induced expressions of these miRNAs associate them with tumor suppressors and oncomirs, respectively. Here, not only do we highlight dysregulated miRNA homeostasis in tumor lesions *per se* and body fluid of cancer patients, but we also discuss advantages and disadvantages of miRNA therapy.

*Address correspondence to this author at the Head, Section for Studies on Metastasis, National Cancer Center Research Institute, 1-1, Tsukiji, 5-chome, Chuo-ku, Tokyo 104-0045, Japan; Tel: +81-3-3542-2511, ex4800; Fax: +81-3-5565-0727; E-mail: tochiya@ncc.go.jp

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1. DISCOVERY OF microRNAs IN CANCER PATHOGENESIS

Functional dissection of miRNAs involved in cancer pathogenesis has generally been begun with miRNA expression profilings, to which various platforms are adapted, including microarrays, TaqMan QRT-PCR, and northern blotting analysis. As a matter of fact, many investigations demonstrated the usefulness of these experimental methods by apparently showing that most of the dysregulated miRNAs in tumor cells can modulate the cell growth, cell invasion or metastasis [17]. Table **1** shows that the mechanisms by which miRNAs are over- or under- expressed widely range from genetic alterations at their own gene loci including deletion, mutation and amplification to epigenetic regulation and malfunction of miRNA processing.

A breakthrough in this research field was achieved by Calin *et al.* in 2002 [18]. This work revealed that the loci of miR-15 and miR-16 on chromosome 13q14 are lost in more than 50 % of B cell lymphocytic leukemias (B-CLL). Furthermore, the same research group checked out the genome loci of 186 miRNAs against cancer-associated genomic regions and fragile sites, 98 of which are coincident with frequently altered regions implicated with cancers [19]. Another successful example is miR-31 which was identified as an anti-metastatic miRNA through expression profiling of 15 non-metastatic and metastatic breast cancer cells [20]. Starting a novel miRNA discovery with miRNA profiling of a set of tumor cells of interest is easy for a large majority of researchers, because it is more difficult and cost-prohibitive to procure human samples of good quality than to prepare *in vitro* research materials. MiR-31 expression levels correlate inversely with metastatic ability of breast tumor cell lines, and the inhibition of miR-31 with a stable miRNA sponge molecule promotes metastasis. Further study revealed that miR-31 blocks several steps of metastasis, including local invasion, extravasation or initial survival at a distant site, and metastatic colonization, whereas cell proliferation is not changed by miR-31. The research group led by Weinberg discovered another metastasis-associated miRNA, called miR-10b, whose expression is at a high level in metastatic breast tumor cells [21]. In the latest review, these metastasis-regulating miRNAs are proposed to be collectively referred to as "metastamir", which is a new category to classify miRNAs by function in cancerous cells [22].

Alternative approach to discover novel functions of miRNAs is a comprehensive genetic screen with miRNA expression libraries. Voorhoeve *et al.* applied a retrovirus-

Table 1. The Mechanisms Causing Dysregulations of MiRNA Expressions

ONC: Oncomir, TS: Tumor Suppressor, CLL: Chronic Lymphocytic Leukemia, AML: Acute Myelogenous Leukemia, MDS: Myelodysplastic Syndrome

Causes for Abnormal Expression	MicroRNAs	Cancers	ONC or TS	Publications
Dysregulated transcription factors				
p53	miR-34	prostate cancer ovarian cancer	TS	[76, 77]
HIF1	miR-210	head and neck tumors	TS	[78]
Epigenetic changes				
promoter region	miR-127	prostate cancer bladder cancer	TS	[79]
Alterations of gene locus				
deletion	miR-15a / 16-1	CLL, prostate cancer myeloma	TS	[18]
	miR-29	MDS, AML	TS	[80]
amplification	miR-17-92	lymphomas multiple solid tumors	ONC	[81]
mutations	miR-15a / 16-1	CLL	TS	[82]
translocations	miR-125b-1	MDS, AML	ONC	[83]
Alterations of processing steps				
LIN-28	let-7	germ-cell tumor hepatocellular carcinoma	TS	[84, 85]
Dicer	miR-204 etc.	ovarian cancer	ONC and TS	[86]
	miR-130b, let-7g	urothelial carcinoma	ONC and TS	[87]

based library for human miRNA expressions to a cell-growth assay of primary BJ fibroblasts which express ecotropic receptor and are immortalized with human telomerase reverse transcriptase subunit [23]. Through the screen for 197 different miRNAs, miR-372 and miR-373 were identified as oncogenes. Further functional analyses showed that these miRNAs negatively regulated the tumor suppressor gene, LAST2, resulting in blocking the p53 pathway. Independently of this study, a trans-well migration assay with an miRNA expression library also revealed that miR-373 enhances metastasis of MCF-7 cells. Together, miR-373 is suggested to function as oncomir as well as metastasis-promoting metastamir [24]. A similar genetic screen approach indicated that miR-221 and miR-222 can promote cancerous proliferation with the ability to inhibit the expression of p27Kip1 tumor suppressor [25].

Two different strategies described above can complement their disadvantages each other. The expression analysis-driven approach helps us easily aware of the physiological relevance of the discovered miRNAs because distorted expressions of miRNAs in cancer cells or patients' tissues could implicate them as a candidate of culprit for the cancer. Hence, the expression signatures of miRNAs must be strictly and accurately evaluated. Given a careless profiling, it would be likely to overlook the physiologically significant miRNAs since miRNAs can induce a wide spectrum of biological events only by the slight difference of their expressions. On the contrary, a comprehensive screen based on the miRNA expression library theoretically enables us to survey all annotated and hypothetical miRNAs with functional assays. However, it would be difficult to elucidate a physiological role of the identified miRNAs without expression profiling data. The past investigations have proved the combination of expression study and functional analysis is a very rewarding approach to identify tumor-related miRNAs.

2. MicroRNAs AS A BIOMARKER

In 2005, Lu *et al.* first demonstrated that miRNA profile reflects the developmental lineage and differentiation state of the tumors through a global analysis of miRNA expression levels in hundreds of clinical samples [26]. Compared with normal tissues, cancerous tissues generally tend to express smaller amounts of miRNAs, suggesting that miRNA signature is a promising tool to classify human cancers [26]. In fact, they were successful in categorizing poorly differentiated tumors using miRNA expression patterns. This report drew investigators' attention to miRNAs as biomarkers for cancer diagnosis. Moreover, a recent literature revealed that miRNA profiling is useful for tracing the tissue origin of metastatic cancers [27]. In this study, the authors employed microarrays to analyze miRNA expression levels in paraffin-embedded and fresh-frozen samples from multiple tumor tissues and metastases. Based on these experimental data, they constructed a biologically driven classification algorithm for identifying tissue origins of metastatic tumors and they demonstrated the accuracy of the algorithm using 65 blinded test samples. In sum, miRNAs have recently been emerging as a versatile biomarker in cancer pathology.

When it comes to clinical applications, it is not practical enough to perform miRNA profiling in patients' biopsies for cancer classification and diagnosis. Of significance is developing easier and less invasive method to early detect cancer lesions. Growing evidence shows the existence of circulating miRNAs in human body fluids and importantly that the amounts of these secretory miRNAs are subject to cancer pathogenesis or development (Table **2**). Lawrie *et al*. reported in 2008 that 3 different miRNAs (miR-155, miR-210, and miR-21) are up-regulated in the serum of patients with diffuse large B-cell lymphoma (DLBCL) [28]. Furthermore, the higher level of miR-21 is well correlated with relapse-free survival of DLBCL patients. In the same year, miR-141 was identified as a circulating biomarker for detection of prostate cancers [29]. The investigators showed another significant finding that the amounts of tumor-specific miRNAs are elevated in the plasma of NOD/SCID mice bearing human prostate cancer 22Rv1 cells, suggesting that the increase in circulating miRNAs in cancer patients can be derived from the tumor cells *per se*. We described in the first paragraph that a tendency to downregulation of miRNA was observed in cancer tissues. In contrast, not only reductions but also inductions were detected in body fluids of cancer patients. There is more to be learnt about the different expression mechanisms between cellular miRNAs and extracellular miRNAs. Our latest issue showed that miR-500 is an oncofetal miRNA in liver cancer [30]. Fetal cells and cancerous cells share several similar features, one of which is that fetal specific genes are observed in tumor cells as well [31]. These so-called oncofetal genes are highly likely to be a specific cancer diagnostic marker because they are not detected in normal adult tissues. MiR-500 is remarkably expressed in a fetal liver, followed by down-regulation in the developmental process, and its expression is in turn recovered in the process of liver cirrhosis. Furthermore, we observed the elevation of miR-500 in the serum of hepatocellular carcinoma patients. Our study sheds some light on secretory oncofetal miRNAs as a potential cancer-diagnostic biomarker.

To establish a minimally invasive diagnostic procedure, the range of surveying new biomarkers is expanding to different bodily fluids and excrements beyond serum and plasma. In 2009, Park *et al.* detected the presence of miRNAs in both the whole and supernatant saliva, and demonstrated that two miRNAs, miR-125a and miR-200a, were lowered in the saliva of oral squamous cell carcinoma patients than in control subjects [32]. On the other hand, a robust method was developed for the preparation of low molecular weight RNA from urine samples, allowing us to evaluate the urinary miRNAs as a tumor marker [33]. Through this attempt, miR-126 and miR-182 are found to overexpress in urine from bladder cancer patients compared with healthy controls. Further discovery of biological materials for diagnosis revealed several distorted expressed miRNAs detected in stool of colon cancer patients with later Duke's stages [34]. Differently from the cases described hereinbefore, feces contain a considerable number of living cells, named colonocytes, which are detached from bowels, leaving open the question which the observed miRNAs are derived from cells or secretory particles.

Since the study on circulating miRNAs has just begun, standard protocols and platforms for evaluating these miRNAs have not yet been established. For instance, the fact that

all investigators do not use the same miRNA as an invariant control miRNA makes it difficult to exactly evaluate different reports. Exogenously spiked-in miRNAs are suitable for normalizing human errors associated with RNA handlings, including extraction and QRT-PCR. But the amount of the spiked-in miRNAs calculated based on the QRT-PCR result does not reflect the total amount of RNA in each sample because the equal amount of the miRNAs is added to each sample. In other words, the difference of RNA amounts between original serum samples cannot be accounted by this normalization method. This problem can be circumvented by designating endogenous miRNAs as an invariant control. As listed in Table **2**, miR-16, miR-638 and RNU6B were previously used for the purpose. However, this approach brings down a new question as to how to define the best invariant endogenous miRNA in each case. To select the secretory miRNA whose expression level is constant under any conditions, it is necessary to repeat a large number of experiments in numerous biological samples.

Table 2. A List of Extracellular MiRNAs whose Expressions are Dysregulated in Human Cancers
Trizol: TRIzol Reagent (Invitrogen), mirVana: *mir*Vana™ miRNA Isolation kit (Ambion)

MicroRNAs	Sources	Dysregulations	Invariant Controls	Platforms for miRNA Measurement	RNA Extraction Methods	Publications
miR-155, miR-21, miR-210	Serum	upregulated in diffuse large B-cell lymphoma	miR-16	TaqMan RT-PCR	Trizol	[28]
miR-141	Serum	upregulated in prostate cancer	cel-miRNAs	TaqMan RT-PCR	mirVana	[29]
miR-141,miR-200	Serum	upregulated in ovarian cancer		Microarray	mirVana	[88]
miR-223,miR-25	Serum	upregulated in non-small cell lung carcinoma	Total amout of RNAs	Solexa, qPCR(stem-loop)	Trizol	[89]
miR-184	Serum	upregulated in squamous cell carcinoma of tongue	miR-16	TaqMan RT-PCR	mirVana	[90]
miR-17-3p,miR-92	Serum	upregulated in colorectal cancer	RNU6B	qPCR	miRNeasy Mini Kit	[91]
miR-17-3p,miR-21,miR-155	Serum	upregulated in non-small cell lung carcinoma		Microarray	mirVana	[92]
miR-29a,miR-92a	Serum	upregulated in colorectal cancer	miR-16	qPCR	mirVana	[93]
miR-21,miR-210,miR-155,miR-196a	Serum	upregulated in pancreatic ductal adenocarcinoma	miR-16	TaqMan RT-PCR	Trizol	[94]
miR-92	Serum	downregulated in acute leukemia	miR-638	TaqMan RT-PCR	Isogen-LS	[95]
miR-500	Serum	upregulated in hepatocellular carcinoma	miR-16	TaqMan RT-PCR	mirVana	[30]
miR-195	Serum	upregulated in breast cancer	miR-16	TaqMan RT-PCR	TRI Reagent BD technique	[96]
miR-125a, miR-200a	Saliva	downregulated in oral cancer	RNU6B	RT-preamplification-qPCR	mirVana	[32]
miR-126, miR-182	Urine	upregulated in bladder cancer	RNU6B	TaqMan RT-PCR	miRNeasy Kit	[33]
miR-21,miR-106a, miR-96, miR-203, miR-20a, miR-326, miR-92	Stool	upregulated in colorectal cancer	18S rRNA	TaqMan RT-PCR	RNeasy isolation Kit	[34]
miR-320, miR-126, miR-484-5p, miR-143, miR-145, miR-16, miR-125b		downregulated in colorectal cancer				

As discussed above, there is no doubt that extracellular miRNAs circulate in the blood in intact structures, despite the presence of RNases [35]. To explain the robustness of circulating miRNAs, it is suggested that some lipoprotein vesicles include miRNAs to protect them from abundantly existing RNases. In fact, endogenous secretory miRNAs are more stable against the treatment of RNases than exogenous naked miRNAs. Moreover, the treatment of some detergents leads to immediate degradation of plasma extracellular RNAs obviously owing to disruption of the lipid vesicles [36]. These findings clearly suggested that extracellular miRNAs are contained in some kinds of secretory particles including apoptotic bodies and exosomes, and thus they are protected from the attack of RNases. However, the underlying mechanism of the secretory process and the biological function of circulating miRNAs are not yet fully understood.

3. SECRETORY PROCESS OF microRNAs

Current investigations have apparently revealed that cancer-specific extracellular miRNAs can be recognized as a promising biomarker in clinical use [37]. In spite of these advances, the following critical and fundamental questions still remain to be solved: tumor-derived miRNAs detected in circulation are as a result of tumor cell death and lyses, or they are actively excreted from tumor cells into the microenvironment, whereby make their way to the blood stream. In this chapter, we present our latest findings of secretory machinery of miRNAs and discuss the sorting mechanism of miRNAs. Our attempt to uncover the miRNA secretory machinery has started with focusing on exosomal miRNAs. Exosomes are small intraluminal vesicles shed from a variety of cells, and their biogenesis is connected with endosomal sorting complex required for transport (ESCRT) machinery in multivesicular bodies (MVB). They are originally considered as a "garbage bag" in order to discard degraded proteins, but now they and their contents, including miRNAs, have generated great interest as an intercellular communication tool. Notably, Trajkovic *et al.* recently reported that exosomes are released independently from ESCRT machinery, but triggered by sphingolipid ceramide [38].

Combining the previous findings and our biochemical and mechanistic analysis, we propose a tentative miRNA secretory process in Fig. (**1**) [39]. MiRNAs are first incorporated into exosomal particles and the surge of cellular ceramide stimulates the release of exosomes "Fig. (**1**)". Ceramide, a major sphingolipid metabolite, is recognized as an important regulator in apoptosis, cell cycle arrest and differentiation [40-42]. Two main pathways have been defined for the generation of ceramide: (1) *de novo* synthesis and (2) the hydrolysis of sphingomyelin by sphingomyelinases (SMase) that are activated under three different pH conditions (acid, neutral, or alkaline) [43]. In *de novo* route, ceramide synthesis begins with the condensation of serine and palmitoyl CoA by the action of serine palmitoyl transferase and then goes on through a series of reducing and acylating reactions. On the other hand, in SMase-dependent synthesis route, the breakdown of sphingomyelin involving the action of this enzyme leads to the formation of ceramide.

Several lines of evidence are emerging to account for pleiotropic biological effects of ceramide. Interestingly, cellular ceramide causes apoptosis in most cancer cells, and this can be accompanied with cell-cycle arrest [44]. Endogenous nSMase2 is up-regulated during confluence-induced growth arrest in MCF7 breast cancer cells. The knockdown experiment with an RNAi technique revealed that attenuated expression of nSMase2 increases cells existing in S phase, resulting from the impeded dephosphorylation of the retinoblastoma protein and the induction of p21WAF1. These results suggest that cellular ceramide can function as a bioregulator of the cell cycle distribution of cancer cells. In contrast, it has remained unclear that secretory miRNAs can be implicated with the ceramide-induced cell growth inhibition. The fact that many kinds of miRNAs can concurrently impact multiple distinct steps of cell growth prompts us to hypothesize that secretory miRNAs can play a significant role in the physiological actions of ceramide.

As illustrated in Fig. (**1**), miRNA secretion depends on ceramide biogenesis, however, the mechanism by which miRNAs are sorted into exosomes for their secretion is not fully understood. Recently, two papers provide the evidence that ESCRT complex associates with components of miRNA effector complexes and RNA silencing takes place on MVBs, suggesting that MVBs could be a crossroads of miRNAs bound for secretory pathway and gene silencing cycle [45, 46]. To support this idea, we presented the data that excretion of miRNAs was not influenced by the knockdown of ALG-2 interacting protein (Alix), a component of ESCRT machinery [39]. This result indicates that secretory pathway can be independent of RNA-induced silencing complex (RISC) activity. To further dissect these intricate routes, it is essential to clarify on which processing step miRNAs are sorted out for their release. In addition to mature guide-strand miRNA, precursor miRNAs and passenger single-stranded miRNAs are also secreted from mesenchymal stem cells [47]. To our knowledge, any primary miRNAs have never been reported to be detected in culture media and biological fluids. These suggest that miRNAs can be sorted out for secretion after the first cleavage in the nucleus. In other words, cytoplasm is highly likely to be the place where miRNAs are assorted, in agreement with the localization of MVB. Further investigations are needed to unveil an intercellular trafficking mechanism by which miRNAs are determined to be secreted or to stay and function in their originating cells.

4. THE ROLE OF SECRETORY microRNAs IN TUMOR MICROENVIRONMENT

Recently, evidence is growing to suggest that secretory microvesicles play a crucial role in intercellular communications. Microvesicles containing EGFRvIII are released to cellular surroundings and blood of glioma-bearing mice, and can merge with the plasma membranes of cancer cells lacking this receptor, leading to the transfer of oncogenic activities, including activation of transforming signaling pathways, morphological change and enhanced cell growth [48]. Between immune cells, exosome vesicles can mediate genetic exchanges as RNAs derived from MC/9 cells are taken up by CD4, MC/9, and HMC-1 cells [13]. These findings inspired us to conceive of the idea that in addition to cytokines, hormones and small molecules, secretory miRNAs emerge as a means of a crosstalk between tumor cells and their surround-

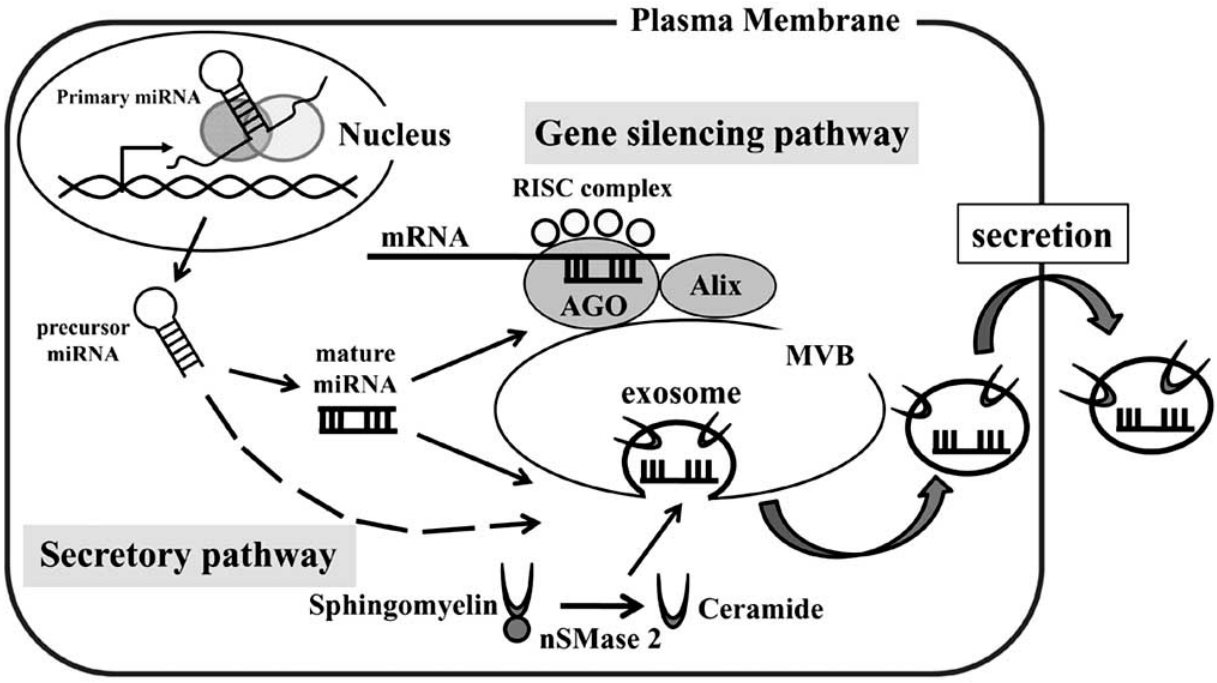

Fig. (1). A tentative sorting machinery of microRNAs.

Cellular ceramide generated by neutral sphingomyelinase 2 (nSMase 2) triggers the secretion of exosome including pre-miRNAs and mature miRNAs. In contrast, compromised integrity of ESCRT machinery blocks miRNA-mediated gene silencing, but it does not affect the miRNA secretion.

ing stromal cells including immune cells, fibroblasts, endothelial cells and bone marrow cells "Fig. (**2**)". Many cancers can expand their territories by moulding their stromal environment to their own advantage [49, 50]. In the context, cancerous cells may evade the attacks of T and B cells by releasing miRNAs to suppress immunoreactions, or may recruit capillary blood vessels with angiogenic miRNAs. Not only do cancer cells cleverly try to tame their neighbor cells by secretory miRNAs, but the surrounding cells can also secrete tumor-suppressive miRNAs so as to block tumor growth and tumor propagation. To underpin this concept, our data show that reintroduction of secretory miR-146a into PC-3M cells where the miRNA is under-expressed attenuated the cell growth [39]. This working model could also help explain the formation of the stem cell niche which offers an interactive environment to maintain a balance among cell proliferation, differentiation, and apoptosis. As long as embryonic stem cells exist in the surroundings, they do not form tumor mass following blastocyst implantation although they share many similarities with cancer cells [31]. In 2009, Giuffrida *et al.* provided evidence that human embryonic stem cells produce secretory unidentified molecules to inhibit cancer cell proliferation [51]. It is likely for extracellular miRNAs to serve as the growth inhibitory signal in the niche surrounding embryonic stem cells. One could envision that the down-regulated miRNAs in cancer cells would be compensated during the initial stage of tumorigenesis by the surrounding cells that could supply exosomes containing the decreased miRNAs. However, once the surrounding cells were not able to meet the demand, cancer cells would lead to an expansive growth. Future studies may show that secretory miRNAs could be conducive to the maintenance and surveillance system against cancer progression.

As miRNAs circulate in body fluids, they could exert hormone-like actions to convey signals to the tissues or organs distant from primary tumor beyond the neighborhood. Importantly, Kaplan *et al.* found that the conditioned medium from the metastatic cancer cells helped in creating a so-called "pre-metastatic niche" where circulating primary cancer cells can easily settle down and form a new tumor mass, thereby dictating organ-specific tumor spread [52]. Further, the authors revealed that the supernatants enhanced the migration of VEGFR1-positive haematopoietic bone marrow progenitors to a future metastatic site and the expression of fibronectin in resident fibroblasts. The cooperation between the newly-recruited bone marrow cells and indigenous stromal cells promotes metastatic spread in specific distant organs. Vascular endothelial growth factor and placental growth factor are listed as a candidate to give rise to these dynamic and systemic changes, however there is no wonder that other tumor-specific humoral factors can be involved in the formation of pre-determined metastatic location. It is assumed that the culture medium with the ability of directing metastasis should contain secretory miRNAs. The key determinant to drive the primary cancer cells to their pre-metastatic niche still remains to be identified.

To delineate the signaling network mediated by secretory miRNAs, not only donor cells but also recipient cells are to be analyzed. As described hereinbefore, the investigation of donor cells focused on secretory machinery is currently making a rapid progress. As for the study on the uptake mecha-

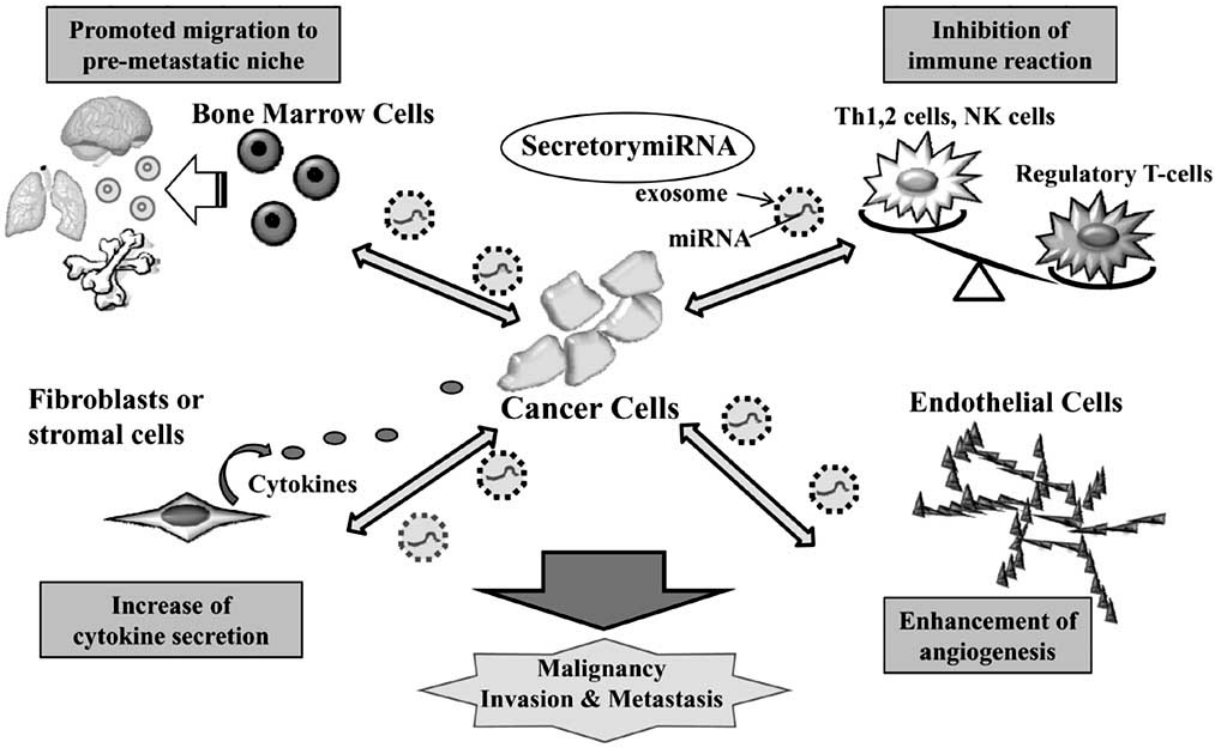

Fig. (2). Secretory microRNAs in tumor microenvironment

Secretory miRNAs act as a communication tool between cancer cells and their surrounding cells.

nism, it was reported that type I transmembrane proteins, T-cell immunoglobulin- and mucin-domain-containing molecule (Tim) 1 and Tim4, can associate with exosome particles [53]. These proteins can specifically bind to phosphatidylserine, which is a major component of exosomes. In sum, Tim1 and Tim4 may be involved in intercellular signaling with which exosomes are implicated. This study paved the way for a comprehensive understanding of a cell-cell communication through secretory exosomes and their contents. Secretory miRNAs may potentially achieve a paradigm shift on the traditional theory of cancer microenvironment establishment.

5. THERAPEUTIC APPLICATION OF microRNAs

5-1. Delivery System

In an attempt to efficiently and tissue-specifically deliver the therapeutic small RNAs, many different approaches are achieved [54, 55]. First, expression vector based delivery can be carried out by using infectious viruses, such as adenoviruses, adeno-associated viruses, or retroviruses [56, 57]. This method can be adapted to an anti-miRNA strategy to knock down specific miRNAs of interest as well. Depletion of miRNAs is implemented with miRNA sponge molecules and miRNA decoy which are transcripts containing multiple tandem complementary sites to the intended miRNA, and therefore they can competitively inhibit the targeted miRNA activity by absorbing the endogenous miRNAs [58, 59]. Alternatively, synthetic small RNAs, including mature miRNA itself and miRNA inhibitor antagonizing specific miRNA, are directly administered into whole bodies with carriers and chemical modifications. Cationic nanoparticles such as lipids, polymers, dendrimers, and cell-penetrating peptides are intensively applied for delivery reagents [55]. They can endow therapeutic small RNAs with abilities to avoid decomposition in the blood stream, penetrate the vascular endothelial barrier, be acquired by the targeted cell and set the small RNAs free into the RNA interference machinery. Two common chemical modifications, 2'-*O*-methyl sugar modification and cholesterol conjugation are used for therapeutic applications [60, 61]. The small RNAs modified by the former method are against endonuclease activity and do not induce as much immune activation mediated by the Toll-like receptors (TLRs) as naked small RNAs [62, 63]. A small RNA duplex whose sense strand is attached to cholesterol at its 3' terminus is readily delivered into hepatocytes through binding to serum albumin [64]. As indicated above, the delivery systems have some potential to improve durability, efficacy and safety, however targeting to arbitrary organs of interest has not yet been succeeded. Of particular note is that most of these findings resulted from preclinical animal experiments. Therefore, further investigations to bridge the difference between mice and humans will be required to accurately assess therapeutic potential and long-term safety of small RNAs.

5-2. Advantages and Disadvantages of microRNA Therapy

We begin this part with clarifying the difference between miRNA therapy and siRNA therapy, which are occasionally confounded even among the scientific researchers in this field. Both strategies take advantage of RNAi machinery to

exert their therapeutic effects, however their concepts and modes of action are distinct each other [65]. Theoretically, siRNA therapeutic application targets on single gene of interest, leading to the knockdown of the gene expression by the degradation of mRNAs. Even if the transcripts of siRNA targeted gene are completely diminished, the subsequent effects on the disease may be limited within a few signaling pathways in which the target gene is involved. Furthermore, since siRNA is an artificially-designed RNA which does not exist endogenously in our bodies, toxicity and off-target effects need to be carefully evaluated for a successful clinical application. Instead, miRNAs, naturally-occurring small RNAs encoded in the genome, can modulate a wide spectrum of gene expressions and cellular signaling cascades. Therefore, severe adverse effects may be less likely and introduction of miRNAs can provide an opportunity to intervene in a variety of processes through disease development. As described in the first chapter, many kinds of miRNAs are aberrantly expressed at reduced levels in cancer progression and development, suggesting that replenishment therapy of the decreased miRNA is suitable for cancer remedy [66].

With respect to negative sides, miRNA therapy has the following concerns: (1) passenger strand effect and (2) resistance to miRNA [66]. Several lines of evidence are currently accumulating to suggest that a passenger strand of miRNAs is regarded as a biologically functional molecule. Recent studies on *Drosophila* miRNAs demonstrated that guide strands and passenger strands are sorted into Argonaute-1 (AGO-1) and Argonaute-2 (AGO-2), respectively [7-9]. Interestingly, AGO-2 is originally identified to serve as a component of RISC mediating the knockdown by siRNAs. The second line of evidence is gained by a global sequence analysis of *Drosophila* miRNAs [67, 68]. This investigation reveals that passenger strands are more highly evolutionarily-conserved than guide strands and that 5'-terminal sequences of passenger strands are more accurately defined than their 3'-ends. Taken together with the fact that "seed sequence", which plays a crucial role in the recognition of 3'-UTR of target gene, is located at 5'-end of miRNAs, passenger strands can potentially exert an miRNA-like regulatory function. Biochemical studies provide the third evidence that some passenger strands can down-regulate the expressions of their predicted target genes in living cells [69-71]. Lin *et al.* shows that miR-199a* (a passenger strand of miR-199a) repressed chondrogenic marker genes, including cartilage oligomeric matrix protein (COMP), type II collagen, and Sox9, resulting in the inhibited early chondrogenesis [69]. Thus, passenger strands of miRNAs are now emerging as a gene expression regulator, not a biologically irrelevant junk molecule. Unfortunately, this finding might be cause for concern about unexpected adverse effects of miRNA therapy because therapeutic miRNAs are administered in a double-stranded RNA form including the desired guide strand as well as its passenger molecule. Biological action of passenger strand in target organs must be scrutinized to evaluate potential side effects of miRNA therapy.

The existence of tumor cells resistant to therapeutic miRNAs is as serious a problem as possible side effects caused by an injected passenger strand. Previous studies show that miRNA binding sites on the 3'-UTR of the oncogenes, including RAS and cyclins, are frequently deleted or mutated in cancer cells [72]. These genetic alterations can impair miRNA-mediated repression, helping tumor cells to escape from the cell growth regulatory system and to gain an extraordinary proliferation ability. This finding indicates that cancerous cells are able to cancel the therapeutic effect of miRNA replacement through altering their own genomic sequences. To succeed in clinical applications of miRNAs, genetic information of their target genes in diseased lesions should be taken into full consideration.

5-3. Development of Diagnostic and Prognostic Tests

At present, miRNAs are likely to be used as biomarkers in clinical settings sooner than as therapeutic reagents. Several commercial companies have already launched their devices and services for the quantification of miRNAs in human body fluids. Exiqon A/S, located in Denmark, has been a leading company offering locked nucleic acid (LNA) technology. LNA-enhanced capture probes are used in their own miRCURY LNA arrays for miRNA detection, enabling the duplex formation with increased thermal stability and improved selectivity. Recently, the technology was applied to QRT-PCR and whereby they improved the accuracy and reproducibility of miRNA measurement in scare clinical samples [73]. An miRNA expression profiling system provided by a German private sector, Febit, adopts a unique on-chip labeling method, termed Microfluidic Primer Extension Assay (MPEA). In this protocol, miRNAs are first hybridized and act as primers for an enzymatic elongation in which biotinylated nucleic acids are incorporated, whereas miRNAs are labeled and followed by hybridization in general procedures. The MPEA improves specificity and sensitivity of miRNA quantification because intact miRNAs without labeling function as a probe in the hybridization step, and thereby even single nucleotide mismatches were recognized with high reliability [74]. Exosome Diagnostics, as the name suggests, is focused on utilizing secretory tumor exosomes to diagnostic markers. They demonstrated that urine exosomes of prostate cancer patients confined two prostate cancer marker genes, *pca-3* and *tmprss2:erg*, indicating that secretory exosomes can represent the tumor transcriptome [75]. These promising venture companies with original technologies and concepts may be the first to develop miRNA detections for cancer diagnosis and prognosis.

6. CONCLUSION

A large amount of scientific reports have been published to show that miRNAs are deeply involved in a wide range of cancer development machineries in many tissues and organs, suggesting that miRNAs will have a significant impact on improving cancer diagnosis and management. On the other hand, many issues still remain to be addressed ahead of clinical use, such as standard platform of miRNA quantification, effective and tissue-specific delivery method, and cost-competitive nucleic acid synthesis technique. Added to this, is a critical question as to whether miRNA therapy can replace the existing clinical applications, including anti-cancer drugs, radiotherapy, and therapeutic antibody. More transparent accumulation of clinical studies comparing between therapeutic miRNAs and existing drugs is needed to answer the question. Taking a realistic view, it is likely that miRNAs

will begin to be clinically employed in conjunction with therapeutic approaches already available. We still have a long and tough way to bring miRNAs from bench to bedside.

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