Ⅲ. 研究成果の刊行に関する一覧表

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Naito Y, Ochiya T	MicroRNA and Hepatitis B, in Book Title: microRNA: from molecular biology to clinical practice	Springer	in press		
Katsuda T, Ikeda S, Yoshioka Y, Kosaka N, Kawamata M, Ochiya T	Physiological and pathological relevance of secretory microRNAs and a perspective on their clinical application.	Biol Chem	395	365-373	2014
Thirion M, Kanda T, Murakami Y, Ochiya T, Iizasa H.	MicroRNAs and oncogenic human viruses. In: Babashah S (ed), MicroRNAs: Key Regulators of Oncogenesis	Springer	pp	155-182	2014
畠 星治、堅田利明、仁科 博史	器官サイズを調節する転写共役因子YAPの活性制御	生化学（日本生化学会）	86	464-468	2014
千葉 恭敬、仁科 博史	肝臓形成および肝がんにおけるHippo-YAPシグナル経路の役割	医学のあゆみ	251	405-409	2014
Yagai T, Miyajima A and Tanaka M.	Semaphorin 3E secreted by damaged hepatocytes regulates the sinusoidal regeneration and liver fibrosis during liver regeneration	American J. Pathology	184	2250-2259	2014
Omi A, Enomoto Y, Kiniwa T, Miyata N and Miyajima A	Mature resting Ly6Chigh natural killer cells can be reactivated by IL-15	Eur. J. Immunology.	44	2638-2647	2014
Kaneko K, Kamimoto K, Miyajima A and Itoh T.	Adaptive remodeling of the biliary architecture underlies liver homeostasis	Hepatology	in press		2015

Morita S, Matsumoto A, Umemura T, Shibata S, Kamijo N, Ichikawa Y, Kimura T, Joshita S, Komatsu M, Yoshizawa K, Tanaka E.	Characteristics and prediction of HBeAg-negative hepatitis following seroconversion in patients with chronic hepatitis B.	Hepatology Research	44	E45-53	2014
Okuhara S, Umemura T, Joshita S, Shibata S, Kimura T, Morita S, Komatsu M, Matsumoto A, Yoshizawa K, Katsuyama Y, Ota M, Tanaka E.	Serum levels of Interleukin-22 and hepatitis B core-related antigen are associated with treatment response to entecavir therapy in chronic hepatitis B	Hepatology Research	44	E172-180	2014

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Review

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Physiological and pathological relevance of secretory microRNAs and a perspective on their clinical application

Abstract: MicroRNAs (miRNAs) have attracted significant attention because of their important roles in a variety of physiological and pathological processes. Recent studies have shown that many cell types secrete miRNAs by packaging them into lipid-bilayered small vesicles called exosomes. Furthermore, exosomal miRNAs travel between cells, exert their RNAi effects in the recipient cells, and play important roles in various biological processes. In this article, we will summarize and describe the latest studies on exosomal miRNAs by focusing on their roles in cancer progression, immune regulation, and tissue repair. We will also provide a perspective on the clinical applications of this research field.

Keywords: cancer; cell-to-cell communication; exosome; microRNA; microvesicle; secretory microRNA.

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Introduction

In the past 20 years, microRNAs (miRNAs) have attracted much attention from various biological fields. miRNAs are small regulatory RNA molecules that modulate the expression of their target genes and play important roles in a variety of physiological and pathological processes, such as development, differentiation, cell proliferation, apoptosis, and stress responses (Bartel, 2009). Since the discovery of miRNAs in *Caenorhabditis elegans* in 1993 (Lee et al., 1993), their presence has been confirmed in many species, including plants (Reinhart et al., 2002) and mammals (Lagos-Quintana et al., 2001). Currently, over 2000 types of miRNAs have been found in the human genome (Table 1) (miRBase ver. 20; http://www.mirbase.org/), and miRNAs are predicted to regulate over one-third of the protein-coding genes (Lewis et al., 2005).

Although miRNAs were first thought to be present and functional exclusively in the cytoplasm, recent studies have shown that they are also present in the extracellular space, for example, in blood, urine, and saliva (Kosaka et al., 2010a). These extracellular miRNAs are protected from RNase degradation, mainly because of encapsulation in lipid-bilayered small vesicles called exosomes. Exosomal miRNAs are not simply cellular byproducts: they are secreted and transported between cells, and they exert their RNAi effects in the recipient cells (Kosaka et al., 2010b; Pegtel et al., 2010; Zhang et al., 2010). Furthermore, exosomal miRNAs play important roles in various biological processes, including cancer progression, immune regulation, and tissue repair.

In this article, we will summarize and describe the latest studies on exosomal miRNAs by focusing on their physiological and pathological roles. We will also provide a perspective on clinical applications of this research field.

miRNAs

miRNAs are non-coding RNAs that are approximately 22 nt in length and that inhibit the expression of various target genes at the post-transcriptional level by binding the 3′ untranslated region of target mRNAs. After transcription from the genome, miRNA genes are processed into mature miRNAs through a two-step incision by Drosha/DGCR8 and Dicer. One of the strands then joins a group of proteins and forms an RNA-induced silencing complex (RISC), which suppresses the expression of target genes (Kwak et al., 2010). The systems are conserved in many species and form important regulators that participate

Table 1 The number of mature miRNAs listed in miRBase.

Species name	miRNA count
Homo sapiens	2578
Mus musculus	1908
Xenopus tropicalis	175
Drosophila melanogaster	426

in multiple biological phenomena, including development, organogenesis, and homeostasis. Because miRNAs can bind to many target mRNAs once their expression is altered, disease can occur through the deregulation of their target gene networks, particularly networks that lead to stress and diseases, such as cancer.

Intercellular transport of exosomal miRNAs and their functions in recipient cells

Exosomes are small vesicles that are released when multivesicular endosomes fuse with the plasma membrane (Théry, 2011) (Figure 1). These vesicles have long been regarded as cellular 'garbage cans' for discarding unwanted molecular components (Théry, 2011). However, exosome research has dramatically changed because of a breakthrough in 1996. Raposo et al. found that exosomes derived from immune cells function as activators of the immune system (Raposo et al., 1996). Subsequently, many groups have reported that exosomes derived from certain cell types contain functional proteins that can activate biological events. These findings have established the novel concept that exosomes serve as a versatile tool for intercellular communication.

The second breakthrough revealed that exosomes shuttle nucleic acids. In 2006, Ratajczak et al. found that exosomes containing mRNA enter target cells, and these mRNAs are translated into the encoded proteins (Ratajczak et al., 2006). The following year, Valadi et al. found that exosomes also contain miRNAs (Valadi et al., 2007). Furthermore, in 2010, three groups independently reported that the miRNAs contained in exosomes travel between cells and suppress the expression of target genes in the recipient cells (Kosaka et al., 2010b; Pegtel et al., 2010; Zhang et al., 2010). Our group reported that

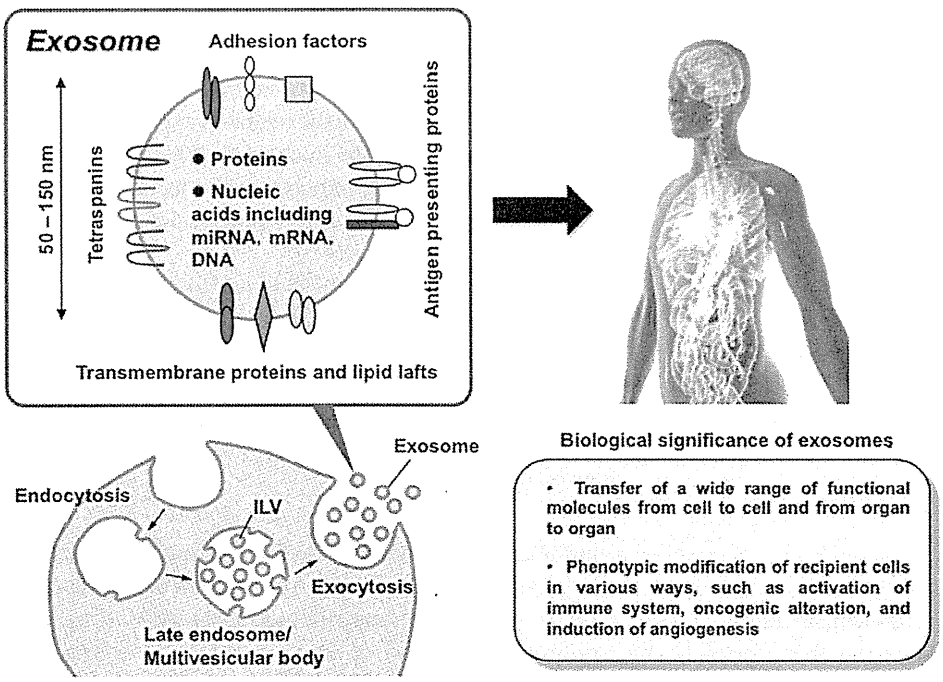

Figure 1 Biosynthesis process, molecular components, and biological significance of exosomes.
During endosomal maturation, intraluminal vesicles (ILVs) are formed through inward budding from the endosomal-limiting membranes. These ILV-containing endosomes are called multivesicular bodies (MVBs) or late endosomes. Some MVBs fuse with the lysosomal compartment and shuttle MVB cargo for degradation, and other MVBs travel to and fuse with the cytoplasmic membrane, where they release cargo ILVs to the extracellular space. These ILVs are secreted via exocytosis and are called exosomes. Exosomes contain a variety of proteins both on the surface membrane and inside the luminal space. Furthermore, exosomes package nucleic acids, including miRNAs, mRNA, and DNA. Recent studies have revealed that exosomes play important roles in various biological events.

a tumor-suppressive miRNA travels between two types of cells and exerts cell growth inhibition. Furthermore, we revealed that the secretion of exosomal miRNAs is dependent on the activity of neutral sphingomyelinase2 (nSMase2), a rate-limiting enzyme of ceramide biosynthesis.

Physiological and pathological relevance of secreted miRNAs

Since the discovery of the functionality of exosomal miRNAs, researchers have investigated the biological significance of secreted miRNAs in a variety of events. miRNAs are well known to be mobile and physiologically functional in plants (Chitwood and Timmermans, 2010), and this has also been found to be the case in mammals. Because miRNAs regulate the expression of various target genes, exosomal miRNAs are likely to serve as a versatile communication tool. Indeed, an increasing number of reports have shown that secreted miRNAs are involved in a wide range of biological processes (Figure 2).

Exosomal miRNAs in cancer biology

The functions of exosomal miRNAs secreted by tumor cells are now of great interest in cancer research (Kosaka and Ochiya, 2011). Since the late 1990s, researchers have explored the involvement of tumor-derived exosomes in cancer development. Early studies identified exosomal proteins that are associated with malignancy. These proteins promote tumor invasion (Higginbotham et al., 2011), promote angiogenesis (Gesierich et al., 2006), and support pre-metastatic and pro-metastatic niche formation (Jung et al., 2009; Peinado et al., 2012). In addition to these proteins, exosomal miRNAs have also been shown to be associated with malignancy in the past few years. In 2008, several groups reported elevated levels of tumor-associated miRNAs in the serum of cancer patients (Lawrie et al., 2008; Mitchell et al., 2008; Taylor and Gercel-Taylor, 2008; Skog et al., 2008). These findings highlighted the relevance of circulating miRNAs in cancer diagnosis. In addition, *in vitro* glioblastoma cell-derived exosomes contain miRNAs, thereby suggesting that they are involved in cancer progression (Skog et al., 2008). Concomitantly, Kogure et al. suggested that a subset of miRNAs enriched in hepatocellular carcinoma (HCC) cell line-derived exosomes can modulate the transforming growth factor β activated kinase-1 (TAK1) pathway (Kogure et al., 2011). Our group recently provided direct evidence that exosomal

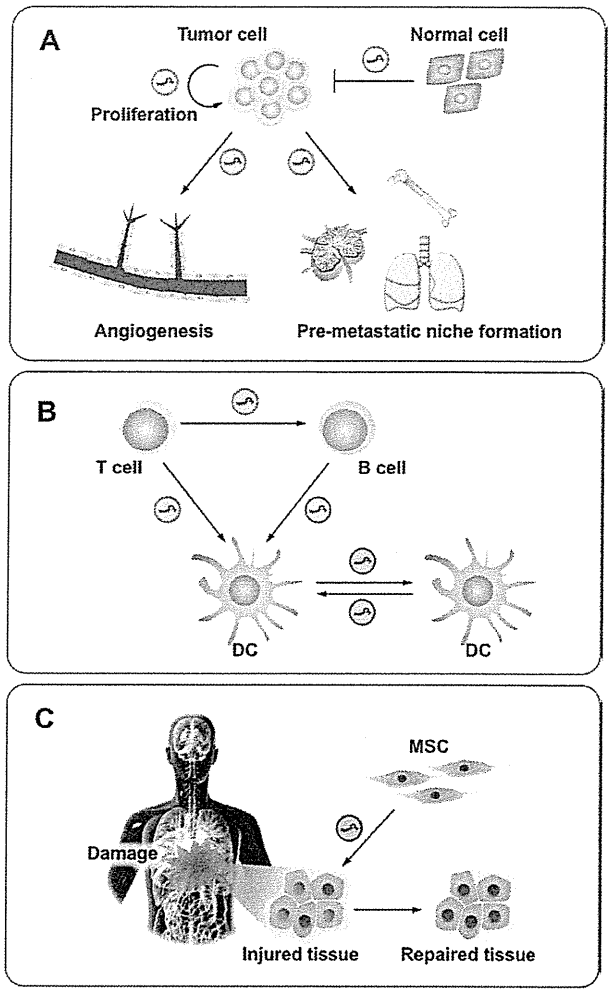

Figure 2 Exosomal miRNAs play important roles in various physiological and pathological events.
(A) Tumor cells produce exosomes containing miRNAs that promote cancer malignancy. These exosomal miRNAs function in several ways. They can promote tumor cell proliferation in an autocrine manner; they can induce angiogenesis, which further supports tumor growth and metastasis; and they can reach distant organs, such as bone marrow, lung, and lymph nodes, thereby promoting pre-metastatic and pro-metastatic niche formation. In contrast, tumor-surrounding normal cells secrete exosomes containing tumor-suppressor miRNAs. (B) Exosomal miRNAs also participate in the immune system. T-cells deliver exosomes containing immune regulatory miRNAs to APCs, including B-cells and DCs. B-cells also transfer exosomal miRNAs to DCs. Meanwhile, DC-to-DC communication, which is essential for amplifying the tolerogenic and immunogenic functions, is partly ascribed to exosomal miRNAs.
(C) In response to damage, MSCs produce and deliver exosomes to injured tissue, which promotes tissue repair. Recent reports have shown that these MSC-derived exosomes contain miRNAs that can promote cell proliferation and suppress apoptosis.

transfer of miRNAs promotes cancer cell metastasis *in vitro* and *in vivo* (Kosaka et al., 2013a). miR-210 released by metastatic breast cancer cells enters endothelial cells

and suppresses expression of its target genes, which results in enhanced angiogenesis. Similar *in vitro* observations have also been reported in leukemia and colon cancer studies (Tadokoro et al., 2013; Yamada et al., 2013). Metastatic cancer cells were suggested to transfer oncogenic miRNAs to non-metastatic cells through exosome release, thereby increasing the malignant phenotype of the recipient cells (Camacho et al., 2013). In addition to the local transfer of miRNAs, several reports have shown that exosomal miRNAs are delivered even to distant organs and contribute to pre-metastatic niche formation (Grange et al., 2011; Rana et al., 2013). Grange et al. showed that CD105-positive renal cancer stem cells release exosomes that contain a set of pro-angiogenic mRNAs and miRNAs. Intravenous injection of these exosomes stimulates angiogenesis in the lung and supports lung engraftment of systemically administered renal cancer cells. Rana et al. also showed that highly metastatic rat pancreatic tumor cell (ASML)-derived exosomes are preferentially taken up by lymph node stroma cells and lung fibroblasts and that they support pre-metastatic niche formation. These authors also suggested that abundant miR-494 and miR-542-3p in ASML-derived exosomes target cadherin-17, which results in the upregulation of matrix metalloproteinase in pre-metastatic lung stroma cells.

In addition to tumor cells, the surrounding normal cells secrete exosomal miRNAs and modulate tumor development. Our group reported that exosomes mediate competitive interactions between cancer cells and normal cells (Kosaka et al., 2012). Exosomal miRNAs secreted by normal cells are transferred to cancer cells and inhibit their proliferation. This finding highlights the important role of normal cell-derived exosomes in the homeostatic mechanism and provides insight into a tumor initiation mechanism. Roccaro et al. reported that exosomes secreted by bone marrow mesenchymal stem cells (BM-MSCs) are associated with the pathogenesis of multiple myeloma (MM) (Roccaro et al., 2013). In contrast to the inhibitory effects of normal BM-MSC-derived exosomes on MM cell proliferation and dissemination, exosomes derived from BM-MSCs that were isolated from MM patients promote MM cell proliferation and dissemination. These authors also reported a decreased level of miR-15a in MM BM-MSC-derived exosomes compared with normal BM-MSCs.

Exosomal miRNA-mediated regulation of the immune system

The first evidence of exosome functionality was provided by immunology research, and intensive studies thereafter have been conducted in this field. In 1996, Raposo et al. reported that B-lymphocytes secrete antigen-presenting exosomes, thereby inducing an antigen-specific major histocompatibility complex class II-restricted T-cell response (Raposo et al., 1996). Following their report, other groups established the concept that antigen-presenting cells (APCs) utilize their exosomes to achieve their role (Zitvogel et al., 1998; Hwang et al., 2003). In addition, mast cell-derived exosomes also participate in immune reactions. Skokos et al. showed that mast cell-derived exosomes activate B- and T-lymphocytes (Skokos et al., 2001) and induce the phenotypic and functional maturation of dendritic cells (DCs) (Skokos et al., 2003).

Exosomal miRNAs also play roles in the immune system. Pegtel et al. demonstrated that exosomal miRNAs secreted by Epstein-Barr virus (EBV)-infected B-cells are transferred to uninfected recipient DCs and that the internalized EBV-miRNAs suppress target genes, such as CXCL11 (Pegtel et al., 2010). The suppression of CXCL11 in DCs may result in dysregulation of the host immune system and allow the EBV to circumvent the immunosurveillance. Exosome-mediated transmission of hepatitis C virus (HCV) has also been reported (Ramakrishnaiah et al., 2013). Of note, exosome-mediated transmission is resistant to neutralizing antibodies, which could explain the ineffectiveness of prophylactic neutralizing antibodies and agents that target the entry of HCV into a cell. Exosome-mediated unidirectional transfer of miRNAs from T-cells to APCs has also been reported (Mittelbrunn et al., 2011). Interestingly, immune synapses between these two cell types are required for efficient miRNA transfer. Exosome-mediated miRNA transfer represents a novel mechanism of DC-to-DC communication, in which DCs interact with neighboring DCs to amplify their functions (Montecalvo et al., 2012). Of note, the pattern of exosomal miRNAs varies according to the maturation of the parental DCs. Compared with immature DC-derived exosomes, mature DC-derived exosomes contain higher expression levels of miRNAs that target pro-inflammatory transcripts in myeloid cells and DCs. This finding supports the idea that exosomes can mirror the phenotype of their parent cell. Another recent report demonstrated the important role of the placenta-derived exosomes in protection from the maternal-fetal spread of viruses (Delorme-Axford et al., 2013). miRNAs that are exclusively expressed in placental cells are packaged within exosomes and transferred to non-placental cells; these miRNAs then attenuate viral replication in the recipient cells by inducing autophagy.

Another remarkable finding regarding the involvement of exosomal miRNAs in the immune system is obtained from reports on breast milk-derived exosomes.

Milk is the only nutritional source for newborn mammals and has unique health advantages for infants. However, the mechanisms through which milk regulates the physiology of newborns are not well understood. Our group first reported that miRNAs are contained in human breast milk (Kosaka et al., 2010c). These miRNAs are encapsulated in exosomes, and they are stable in acidic conditions and resistant to RNase digestion. Notably, among these milk miRNAs, we detected high expression levels of immune-related miRNAs in the first 6 months of lactation, which strongly suggests the role of exosomal miRNAs as novel immune regulatory agents in breast milk. We also confirmed that bovine milk contains immune- and development-related miRNAs. Similar observations have been reported by other groups (Weber et al., 2010; Gu et al., 2012; Zhou et al., 2012; Munch et al., 2013). Interestingly, Munch et al. suggested that the miRNA content in milk can be regulated by the maternal diet (Munch et al., 2013). These authors found that the expression of several miRNAs is altered by a perturbed maternal diet, particularly following a high-fat intake. Furthermore, a recent study has shown that colostrum-derived miRNAs are taken up *in vitro* by macrophages and modulate their immune activities, such as migration and cytokine secretion (Sun et al., 2013). Collectively, although direct *in vivo* evidence is still lacking for the physiological relevance of milk miRNAs, accumulating reports strongly suggest their protective roles against early infections in infants. This hypothesis highlights a novel concept that exosomes can mediate not only cell-to-cell or organ-to-organ communication but also individual-to-individual communication.

Beneficial effects of MSC-derived exosomal miRNAs on tissue repair

Exosomal miRNAs secreted by certain cell types may promote tissue repair in damaged tissue. MSCs reside in mesodermal tissue, such as bone marrow and adipose tissue, and they are attracting much attention due to the therapeutic effects of their secretory factors. These secreted factors are mainly thought to be cytokines and growth factors, but recent studies have revealed that exosomes also contribute to the therapeutic effects of MSCs (Katsuda et al., 2013). In particular, several reports have suggested that miRNAs that are involved in tissue repair are transferred from MSCs to damaged tissue and support regeneration. Chen et al. performed a microarray analysis and found that MSC-conditioned medium contains RNAs of <300 nt encapsulated in exosomes (Chen et al., 2010). These authors also found that exosomal miRNAs are present in a high precursor (pre)- to mature miRNA ratio and that these pre-miRNAs are successfully processed by RNase III to mature miRNAs, thereby suggesting that they are functional in recipient cells. Collino et al. also performed miRNA microarray analysis of MSC-derived exosomes, and they predicted that highly expressed miRNAs in the exosomes could be involved in multi-organ development, cell survival, and differentiation (Collino et al., 2010). These authors performed *in vitro* exosome-transfer experiments, and proteins that were targeted by some of the enriched exosomal miRNAs were downregulated in the recipient cells. Xin et al. found that MSC treatment in stroke model rats results in an increased level of miR-133b, a miRNA that is specifically expressed in midbrain dopaminergic neurons and regulates the production of tyrosine hydroxylase and the dopamine transporter (Xin et al., 2012). The increase of miR-133b and subsequent induction of neurite outgrowth depend on exosome-mediated miR-133b transfer from MSCs to neurons and astrocytes *in vitro* and *in vivo* (Xin et al., 2012, 2013). Additionally, MSCs transfer miR-221 to cardiomyocytes via exosomes and enhance cardioprotection by targeting p53-upregulated modulator of apoptosis (PUMA) (Yu et al., 2013).

Summary and perspective

In summary, accumulating evidence has shown that exosomal miRNAs play versatile biological roles. In the next few years, studies on exosomal miRNAs will provide considerable insight into currently undefined mechanisms underlying various biological phenomena. Simultaneously, translation of these research findings into clinical applications is also a critical issue. Here, we discuss the feasibility of the clinical application of exosomal miRNA research by focusing on two specific topics.

First, the findings that tumor cell-derived exosomes contain oncogenic miRNAs suggest a new direction for cancer therapy. Blocking the secretion of exosomal miRNAs from cancer cells is the simplest idea, but there are hurdles to be overcome. Although several proteins, including Rab family GTPases (Hsu et al., 2010; Ostrowski et al., 2010), nSMase2 (Trajkovic et al., 2008; Kosaka et al., 2010b), and heparanase (Thompson et al., 2013), are involved in exosome secretion, the precise molecular mechanisms have not been fully described. Currently, nSMase2 is most widely accepted as a key molecule in the secretion of exosomal miRNAs and thus is

regarded as a potential target for cancer therapy. Suppression of nSMase2 expression in cancer cells by RNAi technology or using a specific chemical inhibitor, such as GW4869, may provide therapeutic benefit. However, we should be careful about the possible side effects because nSMase2 is involved in a wide range of physiological events, and its deficiency may lead to diseases (Stoffel et al., 2005; Tabatadze et al., 2010; Poirier et al., 2012). In addition to secretory mechanisms, elucidation of the exosomal miRNA-sorting mechanisms will also benefit cancer therapy. If specific molecules recruit oncogenic miRNAs into tumor cell-derived exosomes, such molecules will be a novel target for cancer therapy. Furthermore, several groups have reported that miRNAs can exist in the extracellular space without exosome encapsulation (Kosaka et al., 2013b). In these cases, miRNAs are secreted in association with the RISC effector Ago2 (Arroyo et al., 2011, Turchinovich et al., 2011) or high-/low-density lipoprotein (Vickers et al., 2011). Interestingly, a latest study has reported that EBV-derived miR-BART17 is co-purified with a protein-rich fraction but not with exosomes, whereas miRNA-16 originated from cells is mainly co-purified with the exosome fraction (Gourzones et al., 2013). These exosome-free extracellular miRNAs may also be therapeutic targets, although their biological significance has not yet been documented. In summary, this research field holds great promise for the development of cancer therapies, but extensive further studies are required, especially to elucidate the basic mechanisms underlying the biosynthesis, sorting, and secretion of exosomal miRNAs.

The other possibility for clinical application of this research field is to utilize exosomal miRNAs as drugs. The findings that exosomal miRNAs secreted from MSCs promote tissue repair indicate their potential application for cell-free regenerative medicine. The feasibility of this approach is further supported by the fact that MSCs can be isolated from patients without immunological rejection, and they can be readily expanded many-fold *in vitro*. However, we still must be careful about the safety issues because exosomes contain a wide range of molecules, and it is hard to predict the overall outcome of MSC-derived exosome administration. In particular, we caution that exosomes with tissue-repair effects may serve as an oncogenic factor when non-specifically delivered to uninjured tissue. Thus, it is essential to develop technologies to deliver therapeutic exosomes specifically to target tissue. If surface molecules expressed on the target tissue are known, surface modification of the exosomes will improve the efficiency of specific delivery (Alvarez-Erviti et al., 2011; Ohno et al., 2013). Another potential strategy is the utilization of MSCs as a vehicle for the delivery of exosomal miRNAs. This concept is based on the mouse study by Pan et al., in which it was shown that intrasplenically transplanted Huh7 cells transduced with CD81 shRNA can efficiently deliver functional CD81 siRNA to recipient hepatocytes via exosome transfer (Pan et al., 2011). Because MSCs can be directed and engrafted to injured sites (Chamberlain et al., 2007), the systemic administration of MSCs that have been genetically modified to overexpress therapeutic miRNAs might enable more efficient delivery of exosomal miRNAs than direct exosome administration. Furthermore, if the understanding of exosome secretion mechanisms in MSCs becomes clear, genetic modification of MSCs to increase exosome secretion may enhance the therapeutic feasibility.

In conclusion, research on exosomal miRNAs is now unveiling a variety of biological phenomena whose mechanisms are not yet clear. Furthermore, this research field holds great promise for therapeutic applications, including cancer therapy and regenerative medicine. However, to realize the clinical application, it is necessary to elucidate the fundamental biology of this field, including the mechanisms underlying biogenesis, sorting, and secretion of exosomal miRNAs. Furthermore, we must always be careful about safety issues before clinical applications.

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Chapter 7
MicroRNAs and Oncogenic Human Viruses

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Abstract MicroRNAs (miRNAs) are small, non-coding RNAs that regulate mRNA expression by post-transcriptional mechanism in eukaryotic cells. Some viruses also encode primary transcripts containing miRNA-like structures, and such transcripts are subjected to host miRNA processing pathway to generate viral miRNAs. Viral miRNAs derived from oncogenic viruses are often associated with tumor progression. Moreover, infections with oncogenic viruses alter the expression of host miRNAs, increasing the risk of tumor progression and viral escape from the host immune mechanism. In this chapter, we discuss the roles of virally-regulated cellular miRNAs in the respective viral life-cycles and in virus-related tumors.

Keywords microRNA • Oncogenic viruses • Tumorigenesis • Immune system

1 Introduction

1.1 Discovery of Viral miRNAs

MicroRNAs (miRNAs) are 18–25 nucleotides (nt) non-coding small RNAs derived from double-stranded RNAs, and play an important role in eukaryotic cells by post-transcriptional repression of mRNAs. It has been shown that some viruses encode primary transcripts containing miRNA-like structures. In 2004, Pfeffer et al. (2004) reported that Epstein-Barr virus (EBV) strain B95-8 encodes 5 viral pre-miRNA-like structures, and that viral miRNAs were detected in infected cells. Moreover, Cai et al. (2006) reported that wild-type EBV encodes 13 more pre-miRNAs than EBV B95-8 strain as the 12 kb region that is deleted in EBV B95-8 strain is rich in pre-miRNA genes. The expression of viral miRNAs are very common in cells that are infected

with other Herpesviruses (Pfeffer et al. 2005) (http://www.mirbase.org) including Kaposi's sarcoma-associated herpesvirus (KSHV), human cytomegalovirus, herpes simplex viruses (HSVs), and also observed in simian virus 40-infected cells. It is speculated that viral miRNAs may suppress viral transcripts or host-specific genes. However, the pathophysiological role of viral miRNAs is not clearly understood.

1.2 Viral Infection and miRNAs

Oncogenic viral infections induce the expression of several miRNAs that are associated with cancer progression. Virally induced miRNAs play the role of oncogenes when they target tumor suppressor genes. Moreover, when viral infections involve regulation of oncogenes, they repress some host miRNAs with tumor suppressive functions. Some herpesviruses such as EBV and KSHV encode pri-miRNA-like structures that are tolerated as self-entities by the host machinery. Virally derived-factors repress host miRNA cascade and are called "RNA-silencing suppressors" (RSSs) (de Vries and Berkhout 2008). RSSs were originally identified in plant viruses and oncogenic viruses origin interact with miRNA pathway (de Vries and Berkhout 2008).

2 MicroRNAs in Epstein-Barr Virus; Expression, Regulation and Function Epstein-Barr Virus

2.1 EBV Encoded miRNAs

EBV is a ubiquitous human herpesvirus that establishes life-long latent infection in human B lymphocytes and pharyngeal epithelial cells (kieff 2007). EBV has quite a large genome (~170 kb) and encodes >70 open reading frames. While many of the virally encoded proteins are immunogenic in the human body, miRNAs can affect gene expression in the host without stimulating an immune response. Therefore, encoding miRNAs work to the advantage of the virus. EBV miRNAs were the first virally encoded miRNAs to be identified (Pfeffer et al. 2004). A Burkitt's lymphoma cell line harboring EBV B95-8 strain, a laboratory strain with 12 kb deletion in its genome, (Baer et al. 1984) was used as a source of RNA. Five miRNAs were identified in the study. Later studies revealed that there are far more miRNAs in the wild-type EBV, and the region deleted in the EBV B95-8 strain is rich in pre-miRNA genes (Lo et al. 2012) (Fig. 7.1). Currently, 44 mature miRNAs that are encoded at two different loci in the EBV genome have been identified: 4 mature miRNAs encoded at the BHRF1 locus and 40 mature miRNAs encoded at the BART locus (Pfeffer et al. 2004; Cai et al. 2006; Grundhoff et al. 2006; Zhu

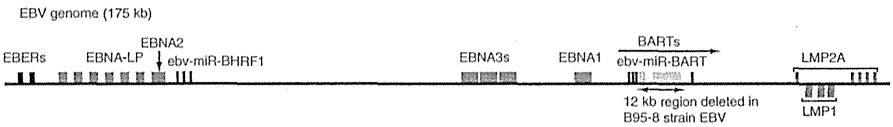

Fig. 7.1 Schematic illustration of EBV genome. The positions of EBV miRNA genes are indicated together with those of EBV latent genes, including BARTs. The 12-kb region missing in the EBV B95-8 strain is also indicated

et al. 2009) (Fig. 7.1). The presence of such a high number of miRNAs in EBV indicates the evolutionary selection of these miRNAs. A complete listing of EBV miRNAs (ebv-miR-BHRF1 and ebv-miR-BART) with both mature and precursor sequences can be found at www.mirbase.org. EBV miRNAs have no notable sequence similarity with known host (human) cell miRNAs, and no orthologous miRNAs are identified in other human Herpesviruses (Pfeffer et al. 2005). In comparison with Rhesus lymphocryptovirus, it is apparent that many of EBV miRNAs are evolutionarily conserved (Cai et al. 2006).

The expression levels of EBV miRNAs in various EBV-infected cells have been examined using various strategies, including the stem-loop PCR method (Amoroso et al. 2011; Chen et al. 2005; Cosmopoulos et al. 2009; Pratt et al. 2009) and direct sequencing of small RNA libraries, either through traditional or high-throughput sequencing method (Lung et al. 2009; Zhu et al. 2009; Chen et al. 2010). The results revealed that EBV miRNAs are expressed at markedly different levels among cell lines (Pratt et al. 2009). Four miRNAs encoded within the BHRF1 locus (hereafter referred to as miR-BHRFs) are highly expressed in cells with latency type III (Xia et al. 2008; Cai et al. 2006) [expressing all EBNAs (EBNA1, 2, 3A-C), LMP1, LMP2A, EBERs, and BARTs (BamHI A Rightward Transcripts)]. The miR-BHRFs are also highly expressed in primary EBV-associated AIDS-related diffuse large B-cell lymphomas (DLBCL) (Xia et al. 2008), but they are undetectable in B cells or epithelial cells with latency type I (expressing EBNA1, EBER, and BARTs) or latency type II (expressing EBNA1, LMP1 and LMP2A, EBER, and BARTs). On the other hand, miR-BART miRNAs (miR-BARTs) are expressed not only in B cells with type III latency, but also in epithelial cells with latency type I or type II (Cai et al. 2006). The miR-BARTs are of particular interest as they are highly expressed in nasopharyngeal carcinomas (Zhu et al. 2009; Cosmopoulos et al. 2009), gastric carcinoma cells (Kim do et al. 2007), and NK/T lymphomas-derived cell lines (Ramakrishnan et al. 2011). Therefore, it is likely that miR-BARTs somehow contribute to the tumorigenesis (Lo et al. 2012; Marquitz and Raab-Traub 2012; Raab-Traub 2012). Transcripts now referred to as BARTs originally identified from nasopharyngeal carcinoma cells (Hitt et al. 1989), have remained enigmatic for many years. However, it is now clear that BARTs most likely serve as primary transcripts that are processed to generate miR-BARTs. Interestingly, the currently identified all miR-BARTs are encoded in the introns of the transcripts of BART, and are subject to highly complicated splicing (Edwards et al. 2008).

Table 7.1 Targeting genes of EBV encoded miRNAs

Target genes	EBV miRNAs	Reference
Viral target genes		
BALF5 (DNA polymerase)	miR-BART2-5p	Barth et al. (2008)
LMP1	miR-BART1-5p, -16, -17-5p	Lo et al. (2007)
	miR-BART9	Ramakrishnan et al. (2011)
	miR-BART19-5p, -5-5p	Riley et al. (2012)
LMP2A	miR-BART22	Lung et al. (2009)
BHRF1	miR-BART10-3p	Riley et al. (2012)
Cellular target genes		
Bim	miR-BART cluster 1 and cluster 2	Marquitz et al. (2011)
PUMA	miR-BART5	Choy et al. (2008)
DICER1	miR-BART6-5p	Iizasa et al. (2010)
CXCL-11	miR-BHRF1-3	Xia et al. (2008)
IPO7	miR-BART3	Vereide et al. (2013)
CASP3	miR-BART16	Vereide et al. (2013)
GUF1, SCRN1	miR-BHRF1-1	Skalsky et al. (2012)
CAPRIN2	miR-BART13-3p	Riley et al. (2012)

2.2 *Pathophysiological Roles of EBV Encoded miRNAs*

Viral miRNAs can either target other EBV transcripts or cellular transcripts. The viral and cellular targets of EBV miRNAs so far identified are listed in Table 7.1. MiR-BART2-5p, which is located directly antisense to the 3′-UTR of BALF5 (a viral polymerase) can down-regulate the expression of BLAF5, inhibiting the transition from latent to lytic viral replication (Barth et al. 2008). Several miR-BARTs suppress the expression of viral oncoproteins LMP1 (Riley et al. 2012; Lo et al. 2007; Ramakrishnan et al. 2011) and LMP2A (Lung et al. 2009). Cellular targets of EBV miRNAs so far identified include proapoptotic proteins Bim (Marquitz et al. 2011) and BBC3/PUMA (Choy et al. 2008), a Dicer (Iizasa et al. 2010), an interferon-inducible T-cell-attracting chemokine CXCL-11/I-TAC (Xia et al. 2008), IPO7, and CASP3 (Vereide et al. 2013). Genome-wide searches for the targets of EBV miRNAs (miRNA targetome) have been conducted using either human Burkitt's lymphoma cell lines (Dolken et al. 2010), primary effusion lymphoma cell lines (co-infected with EBV and KSHV) (Gottwein et al. 2011), or EBV-transformed lymphoblastoid cell lines (Skalsky et al. 2012; Riley et al. 2012).

It is now technically feasible to utilize recombinant viruses, having miRNA genes either deleted or restored in the EBV genome, to clarify the biological significance of viral miRNAs. It was shown, by two independent studies, that disruption of genes encoding miR-BHRF1 results in slightly attenuated outgrowth of infected primary B cells (Feederle et al. 2011; Seto et al. 2010). The EBV B95-8 strain lacks 17 pre-miRNAs of miR-BARTs. Research groups attempted to reconstitute the

expression of all EBV-encoded miR-BARTs by ectopically inserting the missing pre-miRNA genes that were driven by heterologous promoters (Vereide et al. 2013; Seto et al. 2010). However, the displaced miR-BARTs were not expressed as efficiently as the endogenous miRNAs (Seto et al. 2010). The efficient expression of miR-BARTs may require primary transcripts under the control of native BART promoter, followed by proper processing of the primary transcripts.

It was also shown that EBV miRNAs were secreted from infected B cells and that they were functional upon transfer via exosomes in primary monocyte-derived dendritic cells (Pegtel et al. 2010). Another study recently demonstrated that certain plasma EBV miRNAs did not copurify with exosomes, implicating non-exosomal transport of miRNAs into plasma (Gourzones et al. 2013). Further studies are required to clarify the functional significance of viral miRNAs secreted into plasma via exosomal or non-exosomal mechanisms.

2.3 Alteration of Human miRNA Pathway by EBV Infection

Regulating host gene expression is crucial for viruses to survive in host cells, and it is now becoming apparent that viral miRNAs significantly contribute to such regulations, especially in latently infected cells where a few viral proteins are expressed. Viral miRNAs can affect the expression of cellular miRNAs. Specific cellular miRNAs, namely, miR-21, miR-155, and miR-146a, were found to be up-regulated in B lymphocytes transformed by EBV B95-8 strain (Godshalk et al. 2008; Mrazek et al. 2007), while other cellular miRNAs were dramatically down-regulated following EBV infection of primary B cells (Godshalk et al. 2008). It is tempting to speculate that the up-regulation of miR-21 plays critical roles in EBV-mediated transformation, as miR-21 is a well-characterized oncomir (Gabriely et al. 2008). Therefore, it appears that viral and cellular miRNA regulatory networks affect each other, and virus-host interactions are apparently far more complicated than previously thought.

3 MicroRNAs in Kaposi's Sarcoma-Associated Herpesvirus; Expression, Regulation and Function

3.1 KSHV Encoded miRNAs

KSHV belongs to the human herpesvirus family and is implicated in human diseases such as Kaposi's sarcoma (KS), AIDS-related primary effusion lymphoma (PEL), and multicentric castle-man's disease (Boshoff and Weiss 2002). KSHV exists as a latent or lytic infection in host cells. Pfeffer et al. and other groups discovered

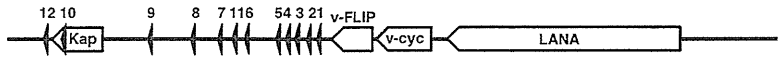

Fig. 7.2 Location of KSHV-encoded miRNAs in KSHV genome *Black triangle*: miRNA, *Kap*: kaposin

KHSV-derived miRNAs in latently infected cells (Pfeffer et al. 2004, 2005; Cai et al. 2005; Samols et al. 2005). KSHV encodes 12 miR-K12 pre-miRNAs (24 miRNAs) and A-to-I RNA edited mir-K12-10a is registered as a mir-K12-10b on miRBase (http://www.mirbase.org) (Pfeffer et al. 2005; Umbach and Cullen 2010; Lin et al. 2010). Most miR-K12s are localized in the intron of K12 (Kaposin) and two pre-miRNAs are localized in the protein- coding region and 3′-UTR of K12, respectively (Fig. 7.2).

3.2 Pathophysiological Roles of KSHV Encoded miRNAs

MiR-K12s are expressed in latently infected cells; however their role in the viral life cycle is largely unknown. MiR-K12-9 suppresses the expression of RTA, which is an essential transcription factor for KSHV lytic infection (Bellare and Ganem 2009; Lin et al. 2011). Transfection of miR-K12-7 or miR-K12-5 also represses RTA-expression (Lin et al. 2011; Lu et al. 2010). Moreover, mutated KSHV that lacks miR-K12s, except miR-K12-10 and miR-K12-12, increased lytic protein expression by enhancing NF-κB activation (Lei et al. 2010). These reports indicate that miR-K12s suppress lytic reactivation and maintain latent infection in host cells.

Seed sequences of miR-K12s are similar to human miRNAs (KSHV-K12-11 and human miR-155, miR-K12-6-5p and human miR-15a and miR-16) (Skalsky et al. 2007; Gottwein et al. 2007). These reports suggest that miR-K12s may target human genes to maintain latent infections. MiR-K12s repressed thrombospondin1 (THBS1), a tumor suppressor, via inhibition of angiogenesis and down-regulation of THBS1 expression, was also previously observed in KS lesion (Samols et al. 2007; Taraboletti et al. 1999). MiR-K12-5, -9, -10a, and -10b repress Bcl-2-associated transcription factor 1 (BCLAF1), which is a repressor of Bcl2 family and induces apoptosis (Ziegelbauer et al. 2009). MiR-K12-11 targets the xCT-negative regulator BACH-1 (Qin et al. 2010a). xCT is an amino acid transporter that protects cells from environmental oxidative stress. KS legions show high expression of xCT (Qin et al. 2010a), and interestingly, xCT is reported to be a regulator of cancer stem cells (Ishimoto et al. 2011). MiR-K12-1 represses cyclin-dependent kinase inhibitor p21. Inhibition of miR-K12-1 results in cell cycle arrest by p53 activation (Gottwein and Cullen 2010). These miR-K12-targeting genes are related to the pathogenesis of KSHV-associated diseases.