RNA extraction. Total RNA was extracted from cultured cells using the QIAzol and miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Real-time PCR (qRT-PCR). The expression of miRNA was quantified by TaqMan miRNA assays (Applied Biosystems, Foster City, CA). The PCR was performed in 96-well plates using the 7300 Real-Time PCR System (Applied Biosystems). All reactions were performed in duplicate. Human-RNU6B or hsa-miR-103 was used as an invariant control.[43]

For the mRNA expression analysis, total RNAs were reverse-transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using a random hexamer primer. The synthesized cDNAs were quantified by TaqMan Gene expression analysis or SYBR Green I qRT-PCR. The β-actin housekeeping gene was used to normalize the variation in the cDNA levels. The primer pairs used for gene amplification are listed in **Supplementary Table S7**.

Quantitative PCR of miR-582 loci on chromosome 5q12. Genomic DNA was extracted from cultured cells using a GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). The qPCR for the miR-582 locus on chromosome 5q12 was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), and the primer sequences were 5′-ccacaacaagtcaatctgtgc-3′ and 5′-tattgaaggggttctggtg-3′. The housekeeping gene RNase P was also quantified as a control reference gene using Platinum Quantitative PCR SuperMix-UDG (Invitrogen) and the TaqMan RNase P Detection Reagents Kit (Applied Biosystems).

Transient miRNA/siRNA transfection. Synthetic hsa-miR-582, hsa-miR-582-5p, hsa-miR-582-3p, and hsa-miR-582-scramble duplexes were obtained from the Bonac (Kurume, Japan). The Allstars Negative Control siRNA was purchased from Qiagen. The siRNAs targeting *KCNC1*, *PGGT1B*, *DIXDC1*, *LRRK2*, and *RAB27A* were purchased from the Bonac. The cells were transfected with 25 nmol/l of either the miRNA or siRNA using DharmaFECT 1 (Thermo Fisher Scientific, San Jose, CA) according to the manufacturer's protocol. The miRNA and siRNA sequences are given in **Supplementary Table S8**.

Cell proliferation assay (MTS assay). For the cell proliferation assay, 24 hours after transfection, 3×10^3 cells were seeded in a 96-well plate. After 3 days of culture, the cell viability was measured using the Tetra Color One assay kit (Seikagaku Kohgyo, Tokyo, Japan) according to the manufacturer's instructions. The absorbance at 450 nm was measured using Envision (PerkinElmer, Norwalk, CT).

Cell invasion assay. The invasive ability of the bladder cancer cells was assayed in 24-well Biocoat Matrigel invasion chambers (8 μm pore size; Becton Dickinson, Franklin Lakes, NJ) according to the manufacturer's protocol. Briefly, the cells were transfected with miRNA or siRNA and, on the following day, 1×10^5 cells were plated in the upper chamber. The lower chamber was supplemented with a medium containing 10% fetal bovine serum. After 24 hours incubation, the cells on the upper surface were scraped off, and the invasive cells attached to the lower surface of the membrane inserts were fixed and stained with Diff-Quik (Sysmex, Kobe, Japan). The invading cells were observed and counted under a microscope in four random fields. All assays were performed in triplicate.

Generation of stable cell lines expressing miR-582 or HA-tagged Ago2 or luciferase. To construct a lentiviral vector for miR-582, pre-miRNA encompassing the stem-loop was amplified from genomic DNA isolated from HT1376 cells by PCR. The PCR product was digested and cloned into the pCDH cDNA cloning lentivector (Cat#CD513B-1; SBI, Mountain View, CA). To construct a lentiviral vector for luciferase, the luc construct was amplified from the pGL4 luciferase reporter vector by PCR. The PCR product was digested and cloned into the pLenti6/V5 Directional TOPO vector (Promega, Madison, WI). The lentiviral vector Lenti-miR-582, Lenti-scramble shRNA (Cat#MZIP000PA-1, SBI), Lenti-HA-Ago2 (RA703B-1, SBI), or Lenti-Luc and the lentiviral packaging plasmids (Invitrogen) were cotransfected into L293T cells. After 48 hours, the lentiviruses in the supernatant were collected and used to infect the UM-UC-3 cells. After antibiotic selection for 2 weeks, stable clones were obtained.

Analysis of miR-582 treatment in a mouse model of bladder cancer. The animal experiments in this study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. For the generation of the lung metastasis mouse model, we referred to some previous studies.[7,44–46] Six- to seven-week-old female Balb/c athymic nude mice (CLEA Japan, Shizuoka, Japan) were anesthetized by exposure to 3% isoflurane on day zero and subsequent days. The murine bladder was injected with UM-UC-3-luc cells intravesically at 5×10^6 cells/50 μl/bladder after 15 minutes of trypsin treatment. The development of subsequent tumor growth and metastasis was monitored once a week by *in vivo* imaging. In brief, the mice were injected with 150 mg/kg D-luciferin (Promega) intraperitoneally and imaged 10 minutes later to count the photons from the whole bladder or lung using the IVIS imaging system (Xenogen, Alameda, CA) according to the manufacturer's instructions. The data were analyzed using the LivingImage software (version 2.5; Xenogen). On day 4, the bioluminescence from the implanted cancer cells was measured, and the mice were divided into two treatment groups with equivalent levels of bioluminescence. The transurethral treatment with the miR-582 and LIC101 (Nippon Shinyaku, Kyoto, Japan) complexes at a ratio of 1:16 (w/w) in a volume of 70 μl (10 μg/site) was performed on days 5, 7, 9 11, 13, and 15.

Ago2-IP. The RNA-binding protein immunoprecipitation was performed using an immunoprecipitation kit (RNA-binding protein immunoprecipitation–assay kit for microRNA; MBL, Nagoya, Japan) following the manufacturer's instructions. In brief, UM-UC-3 cells stably expressing HA-Ago2 were transfected with either miR-582 or NC for 48 hours and immunoprecipitated using anti-HA agarose beads (Wako, Osaka, Japan). The Ago2-bound RNA was eluted from beads with the HA peptide (Wako), and the QIAzol reagent was added to extract the total RNA. Ago2-bound total RNA was cleaned further using miRNeasy columns and then subjected to microarray analysis.

Microarray analysis. Total RNA was harvested from UM-UC-3-miR-582, UM-UC-3-shNC, and UM-UC-3-HA-Ago2 cells that were transfected transiently with miR-582 or NC for 48 hours. The Ago2-bound RNA was prepared from the Ago2-IP experiments. The total RNA was labeled with Cy3 using a Low Input Quick Amp Labeling Kit (Agilent, Palo Alto, CA) and hybridized to a SurePrint G3 Human GE 8×60 K array (Agilent) according to the manufacturer's instructions. The data analysis was performed using GeneSpring GX11.5.

Luciferase reporter assay. The 3′-UTRs of human *KCNC1*, *DIXDC1*, *LRRK2*, *PGGT1B*, and *RAB27A* were amplified by PCR from genomic DNA and cloned at the NotI and XhoI sites into pGMT Easy vector (Promega). The PCR primers and oligonucleotide sequences for the constructs are listed in **Supplementary Table S9**. All the constructs were further confirmed by sequencing.

For the luciferase activity analysis, each construct was cotransfected with the miRNA duplex in a 96-well plate using the DharmFECT Duo transfection reagent (Thermo Fisher Scientific) for 24 hours, and the luciferase assays were performed with the Dual-Glo Luciferase assay system (Promega) according to the manufacturer's instructions.

Western blotting. Forty-eight hours after transfection, the cells were homogenized in an M-PER mammalian protein extraction reagent (Pierce, Rockford, IL). The proteins in the total cell lysate or bound to the anti-HA agarose beads were separated by SDS-PAGE gels, which were calibrated using Precision Plus protein standards (Bio-Rad, Richmond, CA). The primary antibodies against KCNC1 (1: 250, ab84823), DIXDC1 (1: 250,

ab67763), LRRK2 (1: 250, ab57329), PGGT1B (1: 250, ab55615), and RAB27A (1: 200, ab55667) were purchased from Abcam (Cambridge, MA), and the primary antibody against ACTIN (1:10,000, MAB1501) was purchased from Millipore (Billerica, MA). The dilution ratio of each antibody is indicated in parentheses. The HRP-linked anti-mouse secondary antibody (GE Healthcare, Buckinghamshire, UK) was used at a dilution of 1:5,000. The bound antibodies were visualized by chemiluminescence using the ECL Plus Western blotting detection system (GE HealthCare), and luminescent images were analyzed using a LuminoImager (LAS-3000; Fuji Film, Inc., Tokyo, Japan).

Immunohistochemistry. All tumors resected from human bladders were fixed with 10% buffered formalin and embedded in paraffin. Sections with a 5-μm thickness were examined using immunohistochemistry. The sections were deparaffinized, the antigens were retrieved by autoclaving in a 10 mmol/l citrate buffer (pH 6.0), and the endogenous peroxidase activity was blocked with the Immuno Pure Peroxidase Suppressor (Pierce, Chester, UK). The primary antibodies used in this study were the same as those used in the western blotting analysis (1:50), followed by incubation with peroxidase-coupled anti-mouse IgG (ImmPRESS Reagent; Vector labs, Burlingame, CA). The immunoreactions were visualized with diaminobenzidine, and the sections were counterstained with hematoxylin.

Statistical analysis. Results are expressed as the mean ± SE. The statistical analyses were conducted using the Bonferroni multiple-comparison test, and the analyses of the luminescence of the lung were conducted using the nonparametric Mann–Whitney–Wilcoxon test. These analyses were performed with the Expert StatView analysis software (version 4; SAS Institute, Cary, NC). $P < 0.05$ was considered to be statistically significant.

SUPPLEMENTARY MATERIAL

Figure S1. Expression of miR-1305, pri-miR-582 and PDE4D in human bladder cancer cell lines.
Figure S2. Functions of miR-582 in T24 and 5637 cells.
Figure S3. Functional analysis of UM-UC-3-miR-582 cells.
Figure S4. Identification of miR-582-5p and -3p target genes using HA-Ago2 IP and microarray analysis.
Table S1. Copy number losses detected in UM-UC-3 cell line.
Table S2. Copy number losses correlated with pathological stage in human bladder cancer.
Table S3. Stage and Grade distribution of the patients.
Table S4. A total of 259 genes as candidates for miR-582 direct targets.
Table S5. A total of 1,559 genes as candidates for miR-582 indirect targets.
Table S6. Summary of miR-582-5p and -3p target site.
Table S7. PCR primer sequences and oligonucleotide sequences.
Table S8. miRNA and siRNA oligonucleotide sequences.
Table S9. PCR primer sequences for Luciferase assay.

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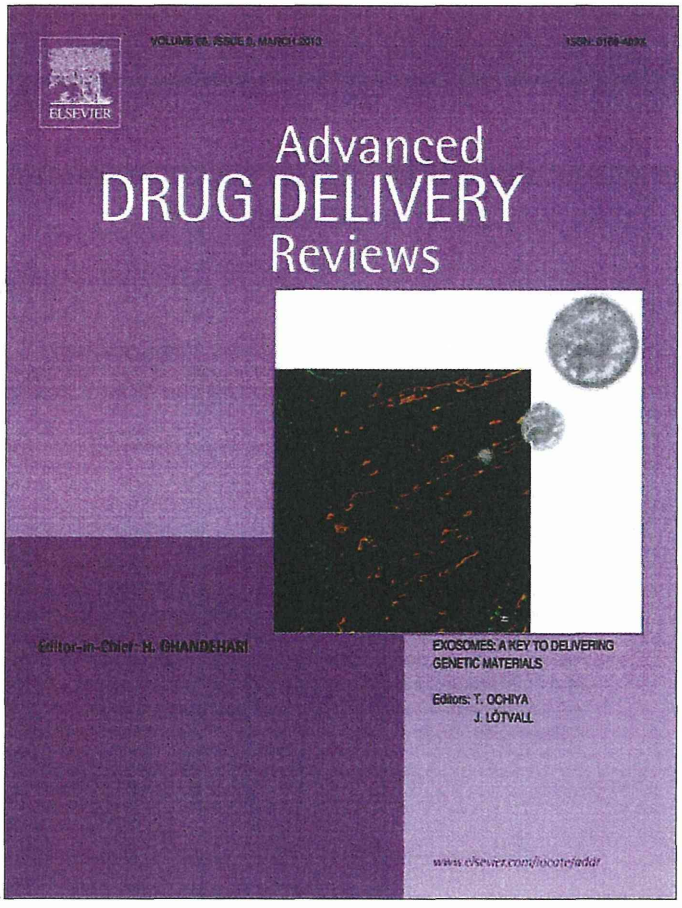

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Exosomal tumor-suppressive microRNAs as novel cancer therapy☆ "Exocure" is another choice for cancer treatment

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ABSTRACT

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

Contents

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1. Introduction

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

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

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

2.1. let-7

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

2.2. miR-16

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

2.3. miR-143

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

2.4. miR-22

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

Table 1
The list of typical tumor suppressive miRNAs.

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

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

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

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

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

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

3.1. The exosomal miRNAs are functional in recipient cells

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

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

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

3.3. The function of exosomal miRNAs in cancer development

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

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

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

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

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

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

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

4.1. Exosomes can be used for siRNA delivery

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

4.2. Exosomes can be used for miRNA delivery

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

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

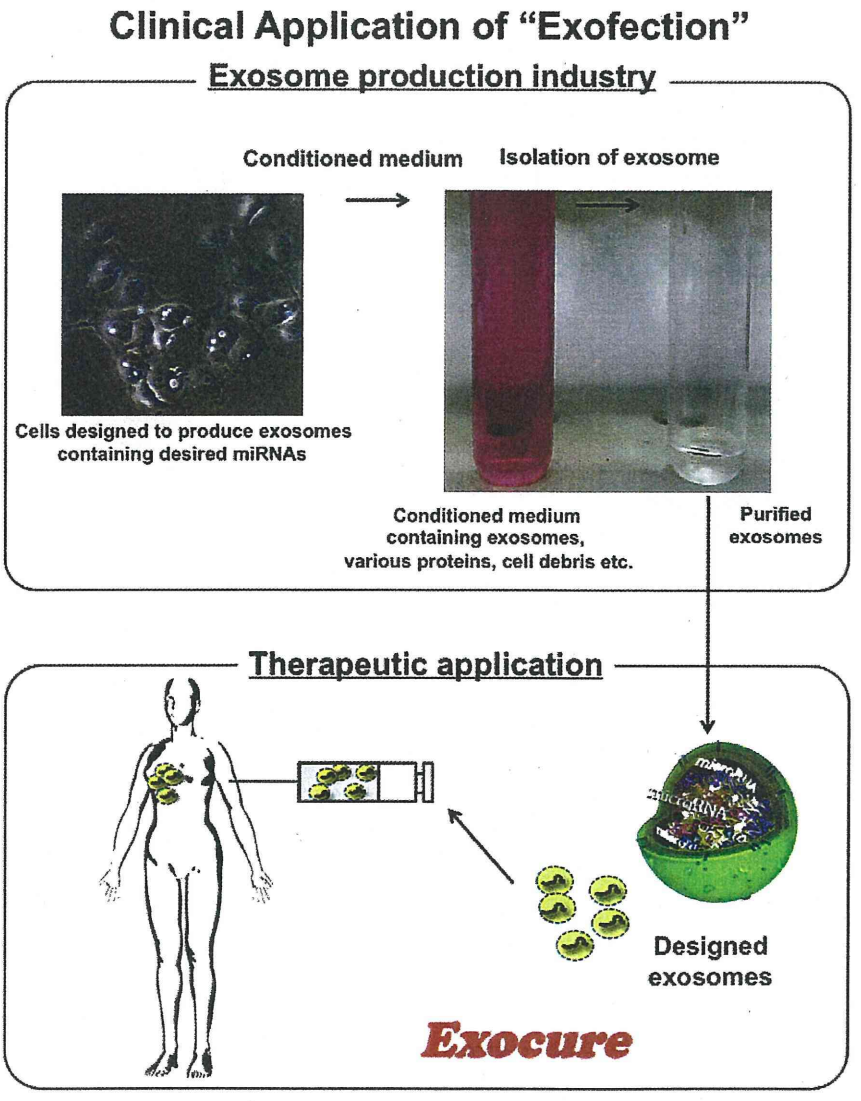

Fig. 1. A schematic explanation of "exocure". Exosome containing desired miRNAs, such as miR-16, miR-143 and so on, is produced by the "exosome production industry", and then these designed exosomes are delivered to cure the patient's disease. For this purpose, some of the issues which are showed in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

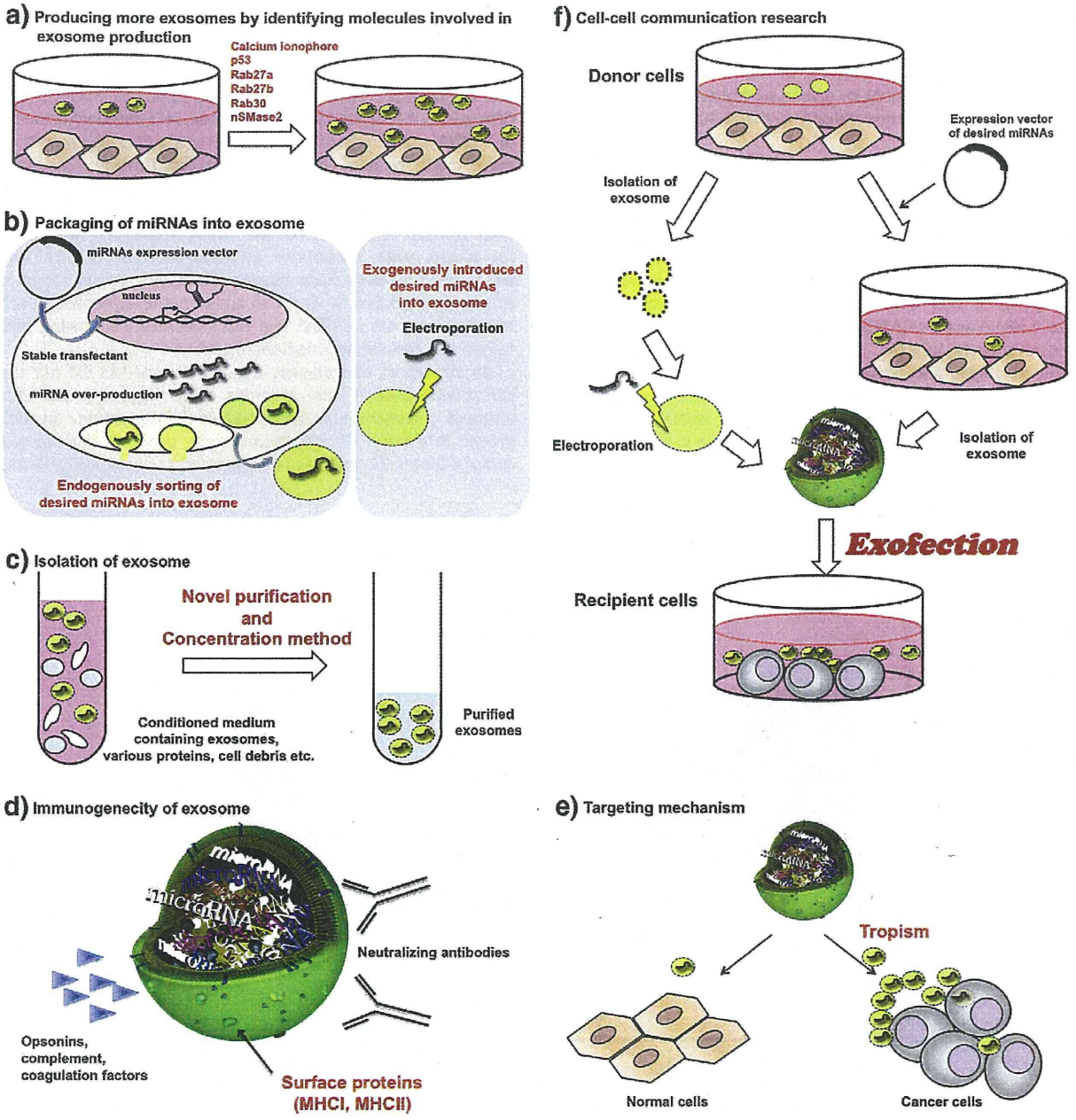

Fig. 2. Issues for the clinical use of "exocure". A schematic explanation of how the novel treatment "exofection" was established and the issues to be solved. To prepare exosomes containing tumor-suppressive miRNAs suitable for clinical usage, clarifying the mechanisms of exosome secretion is important.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

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

5. Perspectives

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

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

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

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