Review Article

Exosomes and other extracellular vesicles-mediated microRNA delivery for cancer therapy

Giuliana Di Rocco*, Silvia Baldari*, Gabriele Toietta

Department of Research, Advanced Diagnostic and Technological Innovation, Translational Research Area, Regina Elena National Cancer Institute, Rome, Italy

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*These authors contributed equally to this work.

Correspondence to: Gabriele Toietta. Regina Elena National Cancer Institute, Department of Research, Advanced Diagnostic and Technological Innovation, Translational Research Area, via E. Chianesi, 53, 00144 Rome, Italy. Email: gabriele.toietta@ifo.gov.it.

Abstract: Extracellular vesicles (EVs), including exosomes and microvesicles, are critical mediators of cell-to-cell communication in tissue homeostasis and repair, both in physiological and pathological conditions. Recently, progress has been achieved in their use in regenerative medicine as transfer agents for active biomolecules. Specifically, EVs are natural carriers of microRNAs (miRNAs), protecting their cargo from plasma ribonucleases and delivering their content to recipient cells. Expression of miRNAs is dysregulated in virtually all forms of cancer. Therefore, EVs-mediated miRNA delivery may represent a valuable tool for cancer therapeutic intervention, aiming at restoring cancer miRNAs expression to normal levels. MicroRNAs may act both as tumor suppressors or oncogenes, consequently different alternative approaches for regulating miRNA expression in tumor tissues have been developed. Here, we review the various strategies for miRNA loading into EVs and highlight studies of EVs-mediated miRNA delivery which have been employed for cancer treatment, both *in vitro* and *in vivo*. Collectively, these data support the use of EVs in miRNAs/miRNAs antagonist transfer for cancer therapy, but challenges related to EV biology have yet to be further addressed before safe clinical translation.

Keywords: Exosomes; extracellular vesicles (EVs); cancer; cell-derived microparticles; microRNA; gene therapy

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Introduction

Extracellular vesicles (EVs)

EVs are membranous vesicles originating from most cells via multivesicular bodies (exosomes), shedding from the cell membrane (microparticles), or produced by apoptotic cells (apoptotic bodies) (1) and released in many body fluids. The nature of the vesicles present in the extracellular environment is quite heterogeneous and consequently several terms have been applied to classify them (2). Discussions regarding the standardization of the isolation procedures and the identification of specific markers have been ongoing for years, but no consensus has been reached on the methods that could be implemented to unambiguously discriminate diverse classes of EVs (3,4). Therefore, in agreement with a previous report (5), we prefer to use the generic term "extracellular vesicles" to collectively denote all the vesicles obtained from biological samples or cell culture supernatants, regardless of the differences in biogenesis and composition.

EVs function as intercellular messengers transferring proteins, RNA species (messenger RNA, long noncoding RNA, microRNA), DNAs (mitochondrial DNA, chromosomal DNA), carbohydrates, and bioactive lipids.

The content of the cargo packaged into EVs differs from that in the originating cells, indicating that the loading process is selective (6). Circulating EVs can, therefore, represent powerful, minimally invasive, specific diagnostic and prognostic biomarkers for numerous diseases, including cancer (7,8). On the other hand, delivery of therapeutic bioactive molecules, in particular miRNAs, through EVs may be an innovative avenue for cancer therapy (9-12). In facts, as delivery vehicles, EVs support the release of microRNAs, anti-miRNA oligonucleotides and small interfering RNAs, protecting them from degradation. Due to lack of class I/II MHC molecules expression, EVs isolated from mesenchymal cells do not promote immune response, thus allowing harvesting also from non-autologous cells and repeated administration (13). Moreover, EVs have negligible toxicity and can deliver their cargo across the blood-brain barrier (14).

Micro RNAs

Micro RNAs (miRNAs) are a class of approximately 22 nucleotide-long, non-coding RNAs predominantly involved in the regulation of target gene expression, mainly at post transcriptional level, by binding to a complementary mRNA sequences (15). More than 2,500 miRNAs have been identified in eukaryotic cells, and they have been demonstrated to play a pivotal role in regulating diverse physiological and pathological processes including cancer development, metastasis and drug resistance (16,17).

Extracellular miRNAs have been identified in several biological fluids (18), protected from RNase degradation by association with Argonaute (Ago) proteins (19), in high- and low-density lipoprotein particles (20) or by inclusion into extracellular microvesicles (10). Up to 99% of circulating plasmatic miRNAs are bound to an Argonaute protein and are not enclosed within EVs (21). Argonaute are highly conserved and ubiquitously expressed proteins which play a key role in gene silencing. Ago proteins bind small non coding RNAs, including miRNAs, and mediate repression of specific target genes promoting mRNA degradation or inhibiting translation (22). Circulating miRNAs are mainly produced as "trash" RNAs from cells undergoing damage, necrosis and apoptosis. The functional role of this heterogeneous population of extracellular miRNAs is still a matter of debate (23,24). Yet, several lines of evidence strongly suggest that functional miRNAs contained into EVs can be delivered to, and specifically regulate gene expression on distal target cells during viral infection, immune response and tumor progression (25,26).

The ability of EVs to naturally and effectively transfer genetic information has opened up the perspective exploitation of EVs-mediated miRNAs delivery to modulate target genes expression for therapeutic purposes (27). EVs are less immunogenic and toxic in comparison to other gene therapy vectors, making them an ideal tool for local and systemic delivery of small RNA-based therapeutic molecules (11,28,29). To this end, different strategies have been developed for introducing exogenous genetic material, including miRNAs and anti-miRNAs, into EVs produced by non-tumoral cells (30).

Strategies for RNA loading into EVs

Less than 10% of the RNAs present into EVs corresponds to the transcripts in the originating parental cells (27), suggesting the existence of specific cellular machineries for loading RNA into EVs (31,32). The majority of the endogenous genetic material present into EVs is highly fragmented, with sequences below 700 nucleotides in length (33); a similar size limitation cutoff has been observed in studies of exogenous DNA packing into EVs (34). Therefore, EVs are unsuitable for full coding sequences transfer, but they can easily allocate and protect from endonuclease degradation non-coding miRNAs and siRNA, which are approximately 18–25 base pairs in length (26). Mechanisms of selective sorting are currently poorly characterized, but the packaging of miRNA into EVs seems to be correlated with the presence of a specific GGAG sequence, named EXOmotif (35,36).

EVs can be purified from conditional medium collected from large-scale *in vitro* cultures of producing cells such as mesenchymal cells (37). The yield, purity and integrity of RNA recovered from EVs are influenced by the methods of EVs purification (38). Therefore, precise analysis and accurate quantification of RNA in EVs is a challenging task, further complicated by the lack of specific standards. Notwithstanding these limitations, it has been estimated that, on average, in a mixed population of purified EVs there is less than one molecule of a given miRNA per EV (39), suggesting that either only a yet to be identified subpopulation of vesicles contains significant amounts of miRNAs or very few miRNA molecules are present in a single EV (40). At any rate, this level seems inadequate for effective target gene modulation (23). On the other hand, a single cell may produce up to 30,000 EVs per day and up to 500 copies of miRNAs can be loaded into a single vesicle (41).

Table 1 Methods for RNA loading into EVs

Methods	Ref.
Pre loading strategy	
Transfection of miRNA mimics	(45-49)
Transfection of plasmidic vector expressing specific miRNAs	(50)
Electroporation of plasmidic vector expressing specific miRNAs	(51)
Viral mediated transfer of miRNA coding sequence with EXOmotif	Baldari (u.r.)
Post loading strategy	
Transfection plus heat shock of miRNA mimics	(52)
Electroporation of miRNA mimics	(53-56)
Virus modified EVs	(41)

u.r., unpublished results; EV, extracellular vesicle.

Therefore, by re-engineering naturally-derived EVs to increase specific miRNA payload, it might be feasible to obtain a suitable amount of EVs for clinical anti-tumor therapies (42-44). Two main approaches for loading miRNAs into EVs have been developed (*Table 1*) (57): the first, referred as preloading or endogenous method, involves the genetic modification of the EVs-producing cells; in the second, named post-loading or exogenous method, miRNAs are loaded into previously purified EVs (58). Each loading strategy has its advantages and limitations; the type of cells used as EVs source and/or the nature of the genetic material to deliver ultimately dictates the method of choice. For instance, by the pre-loading approach, using stable transfection or viral-mediated gene transfer, it is possible to establish genetically modified cell lines as convenient and dependable source for the *in vitro* production of EVs. On the other hand, transfection of primary cells with high efficiency and low toxicity, either with plasmid DNA, synthetic oligonucleotides or viral-mediated gene transfer, may represent a challenging task making the post-loading approach more suitable.

Pre-loading approach

The preloading approach is based on the observation that cellular overexpression of a selected miRNA determines the increment of its content into EVs (59). EV-producing cells can be either modified through genetic engineering using miRNA expressing plasmid or viral vectors or by introducing exogenous synthetic oligonucleotides that mimic the function of natural miRNAs, which are subsequently incorporated into secreted EVs. Possible interference of transfection reagents with EVs packaging should be taken into account (60). Our understanding of the cellular mechanisms of miRNAs sorting into EVs is incomplete; therefore, it is not clear whether some families of miRNAs might be preferentially packaged into EVs, making this method suitable only for some classes of miRNAs (61,62). Moreover, it should be pointed out that in the cell culture supernatant the majority of miRNAs is not associated with EVs (23); consequently, the efficiency of miRNA loading into EVs is generally limited. In addition, the pre-loading method requires extensive optimization in each distinct cell type used as EVs source.

Post-loading approach

In the post-loading approach synthetic miRNA oligonucleotides are introduced by electroporation or transfection into previously isolated EVs. Potentially, this should be a more controlled process compared to the pre-loading approach, but increased problems related to EVs and miRNA integrity and functionality may arise (63). RNA precipitation and aggregation in the electroporation buffer have been observed during the process of transfer of siRNA oligonucleotides into EVs, making difficult to evaluate the loading efficiency, estimated to be below 0.05% (64). Refinement of the experimental condition permitted to achieve a 55% efficiency of miRNA loading into EVs (53). In contrast, other researchers have reported inefficient EVs loading of miRNA by electroporation and have opted for techniques based on transfection (65). Recently, a calcium phosphate co-precipitation transfection plus heat shock method for introducing miRNAs into isolated EVs has been reported (52). Then again, caution should be exercised in determining the loading efficiency because of the possible presence of complexes between transfection reagents and miRNAs not enclosed into EVs. The discrepancy in the success rate of miRNA loading reported by different groups may be further attributed to differences in EVs producing cells and/or in EVs purification methods.

EVs for therapeutic microRNA delivery

The use of EVs-mediated miRNA delivery to modulate target genes expression is currently under investigation as a beneficial tool for cancer therapeutics (66-68). Expression

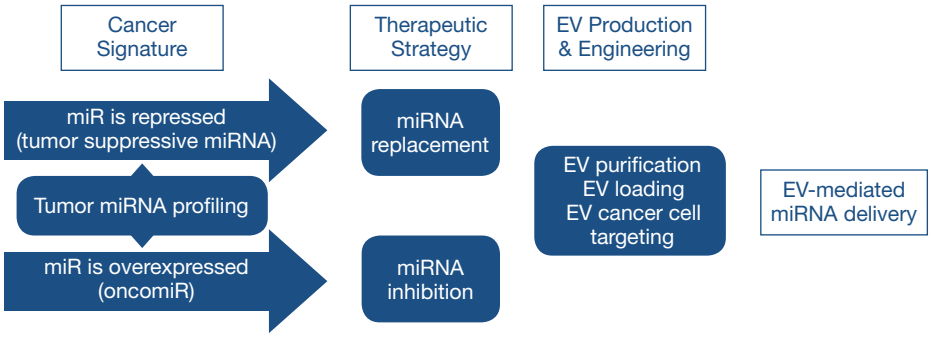

Figure 1 Development of extracellular vesicles-mediated miRNA delivery strategies for cancer therapy.

of miRNAs is aberrant in practically all forms of cancer (17); therefore, reversion to normal levels is a potential way for therapeutic intervention (69). Targeting specific molecular pathways, microRNAs may act both as tumor suppressors or oncogenes (70,71); consequently, two alternative strategies are intended to reestablish physiological miRNA expression in tumor tissues, either by restoring or repressing miRNA activity (*Figure 1*) (72).

miRNA as therapeutic agents: EVs-mediated miRNA replacement therapy

Some miRNAs act as tumor suppressors promoting cancer development through down regulation of cellular oncogenes. The expression of tumor-suppressor miRNAs is lower in cancer compared to normal adjacent tissues. Therefore, restoring normal levels by miRNA replacement strategy may provide therapeutic benefit (73). Exogenous administered miRNAs are expected to function as the endogenous counterparts, minimizing the risk of off-target effects. However, it cannot be ruled out that reaching supra physiological levels, exogenously administered miRNAs may target also previously unidentified genes (11). In addition, exogenous administration of miRNAs may lead to the saturation of endogenous miRNA processing enzymes, possibly leading to perturbation of miRNAs function (72,74). Furthermore, circulating miRNAs, in addition to their canonical role as post-transcriptional modulators, may act as ligands of toll like receptors, resulting in pro-tumoral stimulation (75).

miRNA as therapeutic target: EV-mediated miRNA inhibition therapy

The miRNAs overexpressed in cancer are considered as oncogenes and are consequently denoted as oncomiRs (71). They promote tumor development by down regulating tumor suppressive genes or genes implicated in cell differentiation or apoptosis. Therefore, the miRNA inhibition therapeutic strategy aims at inhibiting oncomiRs' expression by delivery of specific miRNA antagonists, such as anti-miRNAs, locked-nucleic acids (LNA), or antagomiRNAs (76,77).

Preclinical studies

Growing preclinical data support innovative EVs-based approaches for cancer therapy (1,42). Here we review the most recent preclinical studies which employed miRNAs or anti-miRNAs enclosed into EVs as active agents against various types of cancers (*Table 2*).

EVs naturally released by adult liver stem cells may inhibit the growth of hepatoma cells *in vitro* and *in vivo* via miRNAs transfer (80). In addition, several *in vitro* studies have clearly demonstrated that miRNA loading into EVs, and subsequent EVs-mediated miRNAs transfer can modulate gene expression in distinctive tumor target cells including breast cancer cells (45), and osteosarcoma cells (46). Unpublished results obtained by our group suggest that lentiviral vector-mediated transfer of EXOmotif-containing miRNAs into adipose tissue-derived stromal cells may represent a suitable strategy to obtain stable production of EVs for miRNA replacement therapy. Additional studies have provided the proof-of-principle of an effective reduction of tumor growth upon treatment with miRNA-loaded EV in different animal models. For instance, EVs harvested from cell culture supernatants of adipose tissue-derived stromal cells transfected with a plasmid expressing miRNA-122 were able to increase hepatocellular carcinoma cells sensitivity to Sorafenib both *in vitro* and in a xenograft

Table 2 EV-mediated miRNAs or anti-miRNA delivery for cancer therapy

Cargo	EV source	EV isolation	Tumor target	Ref.
MiRNA delivery				
MiR-122	AT-MSCs	ExoQuick-TC	Hepatocellular carcinoma	(50)
MiR-134	Hs578Ts cells	ExoQuick	Breast cancer	(45)
MiR-143	BM MSCs	Differential centrifugation	Osteosarcoma	(46)
MiR-146b	BM MSCs	ExoQuick-TC	Glioma	(51)
Let-7a	HEK293 cells	Differential centrifugation	Breast cancer	(65)
Let-7a	Dendritic cells	Differential centrifugation	Breast cancer	(55)
MiR-143	Macrophages	–	Colon cancer	(47)
MiR-125b	AT-MSC	ExoQuick TC	Hepatocellular carcinoma	Baldari, u.r.
Anti-miRNA delivery				
Anti miR-9	BM MSCs	Differential centrifugation; total exosome isolation kit	Glioblastoma multiforme	(78)
Anti miR-150	HEK293 cells	Differential centrifugation	Sarcoma	(79)

ExoQuick TC, exosome precipitation reagent (System Biosciences, Mountain View, CA, USA); total exosome isolation Kit (Invitrogen, Carlsbad, CA, USA). u.r., unpublished results; EV, extracellular vesicle.

Table 3 Clinical trials investigating extracellular vesicles delivery in cancer therapy

Indication	EVs source	Phase	Ref.
Metastatic melanoma	Dendritic cells pulsed with antigen peptides	Phase I	(86)
Colon cancer	Ascites	Phase I	(87)
NSCLC	Dendritic cells pulsed with antigen peptides	Phase I	(88)
NSCLC	Dendritic cells	Phase I-II	NCT01159288* (89)
Malignant ascites and pleural effusion	Tumor cell derived EVs loaded with chemotherapeutic drugs	Phase II	NCT01854866*
Colon cancer	Plant exosomes conjugated with curcumin	Phase I	NCT01294072*

*, ClinicalTrials.gov Identifier. NSCLC, non-small cell lung cancer.

model (50). Similarly, EVs isolated from bone marrow-derived stromal cells overexpressing miR-146, delivered via intra-tumor injection, reduced primary brain tumor growth in a rat xenograft model (51). Modifications of EVs to confer tumor targeting ability have been evaluated, allowing for specific therapeutic delivery of microRNA to breast cancer cells (55,65).

Also EV-mediated miRNA inhibition therapy has been proven effective in experimental models of glioblastoma multiforme (78) and sarcoma (79), specifically downregulating the expression of miR-9 and miR-150, respectively.

Current challenges to the clinical translation

EVs have been recognized as paracrine mediators of cell-based therapy, generating interest on their use in clinical applications for regenerative medicine purposes (81-83). In recent years, the potential of EVs as vehicles for the therapy of cancer has also been proposed (84,85), but published results of trials using EVs as miRNA carriers are not available yet (*Table 3*).

In particular, clinical trials investigating EVs delivery for cancer treatment has mainly focused on the use EVs derived from dendritic cells for tumor immunotherapy (86,88). Additional studies have investigated EVs for delivery

of chemotherapeutic drugs (*Table 3*). Collectively, these phase I and II trials provided evidences on the feasibility of producing clinical grade EVs and on the safety of their administration with no adverse effects observed (90).

Several clinical trials using microRNAs as therapeutic agents are currently under investigation (91,92); successful clinical application requires the development of an effective and safe delivery system (29). EVs are naturally-adapted transporters of miRNAs with negligible toxicity, low immunogenicity, high stability, and which are also amenable to modifications aimed at conferring tropism as well as at improving the loading of specific cargoes (84,93). Nonetheless, several issues need to be addressed before clinical use of EVs as therapeutic tools (58). Crucial problems are: (I) identification of the most proficient EVs cellular source suitable for clinical application; (II) optimization of the methods for obtaining high yields of pure EVs; (III) definition of the requirements for the characterization of purified EVs; (IV) setting of a regulatory framework for using EVs for therapeutics as advanced therapy medicinal products (58). Additional specific problems arise for the clinical translation of EVs-mediated miRNA delivery for cancer therapy (85). In particular, methods for miRNA/antagomiRNA loading into EVs (94) and for precisely defining the yield of the loaded cargo should be optimized (38). A better understanding of the molecular mechanisms by which miRNAs are sorted into EVs and subsequently released by different cell types is needed (95). Purified EVs loaded with miRNAs/anti-miRNAs can be delivered either systemically or through local injection into the tumor (72). For therapeutic intervention on easily accessible primary tumors such as melanoma or breast cancers, EVs can be delivered by local administration, reducing the risks of dissemination, off-target effects and toxicity. However, for other tumors and for metastatic cancers, EVs should be delivered systemically. Though, preclinical studies suggest that exogenously EVs administered by systemic administration are rapidly cleared by the macrophages of the mononuclear phagocyte system (81,96). A deeper understanding in biodistribution and pharmacokinetics profiles of EVs administered by different routes and modalities (acute *vs.* repeated administration) is required before clinical translation (56,81). Interestingly, some preclinical studies have provided evidence that EVs can be engineered to enhance their targeting capability to tumor tissues (65). Cellular uptake of EVs is likely to be cell type-specific, however the mechanisms involved in the process are not completely elucidated (97). In addition, dose escalation studies of exogenously administered EVs loaded with miRNAs should be performed in order to define the therapeutic window and the maximal dose permitted without saturating the endogenous miRNA processing machinery in non tumoral cells (72).

Conclusions

EVs represent a safe vehicle for efficient delivery of therapeutic miRNAs. Strategies to further promote the therapeutic efficacy of EVs, such as methods for loading specific miRNAs/anti-miRNAs and to provide for targeted uptake by tumor cells have been proven effective in preclinical studies. Processes of pharmaceutical manufacturing of EVs suitable for clinical application are currently under investigation (98). However, before clinical application of EVs mediated miRNA delivery as a therapeutic strategy in cancer, a better understanding of EV biogenesis and functions is needed.

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Footnote

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