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Extracellular vesicle-based targeted RNA therapies against cancer

Ziqi Wang [a], Haonan Xing [c,*], Yuanyu Huang [a,b,**], Mei Lu [a,b,***]

[a] School of Life Science, School of Interdisciplinary Science, Key Laboratory of Molecular Medicine and Biotherapy, Key Laboratory of Medical Molecule Science and Pharmaceutics Engineering, Beijing Institute of Technology, Beijing, 100081, PR China
[b] School of Medical Engineering, School of Interdisciplinary Science, Affiliated Zhuhai People's Hospital, Beijing Institute of Technology, Zhuhai, 519088, PR China
[c] Chinese Academy of Sciences (CAS) Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, CAS Center for Excellence in Nanoscience, National Center for Nanoscience and Technology of China, Beijing, 100190, PR China

A B S T R A C T

Extracellular vesicles (EVs) are nanoscale vesicles released by cells and serve as natural carriers for RNAs, DNAs, proteins, and lipids that mediate intercellular communication. EV application as nanocarriers in targeted cancer therapy has gained significant attention. The delivery of RNAs via EVs has emerged as a promising technology in the past few decades, as EVs can encapsulate RNAs to protect them from degradation and enhance their uptake by recipient cells. Notably, chemical or genetic modifications to the surface of EVs can further strengthen their targeting ability. These advancements not only improve the specificity of RNA therapies but also address the challenges of RNA delivery associated with traditional methods. This review discusses the recent advancements in the delivery of messenger RNAs (mRNAs), miRNAs, siRNAs, and other RNA species for targeted cancer therapy via EVs. We aim to provide critical insights into the strategic design of advanced EV-based nanoplatforms for RNA delivery.

1. Introduction

According to a report released by the American Cancer Society (ACS), approximately 20 million new cancer cases were diagnosed in 2022, with around 9.7 million deaths attributed to cancer, making it one of the leading causes of death globally.[1] Current treatment options include radiotherapy, chemotherapy, and surgical interventions. To mitigate the adverse effects of radiotherapy and chemotherapy, a lot of new cancer treatments have been developed. Cancer immunotherapy has aroused extensive interest with numerous products being commercially available, such as nivolumab and pembrolizumab.[2,3] However, a major challenge of immune checkpoint inhibitors is the low immune response rate in the clinic,[4] highlighting an urgent need for safe, effective, and precise treatment options for the majority of patients. Since the targeting ability determines the therapeutic effect to some extent,[5] targeted tumor therapy has attracted widespread attention. These therapies act on specific targets within tumors, exerting effects primarily on tumors and related cells while minimally impacting healthy cells, which may achieve favorable therapeutic outcomes with reduced toxicity. Specifically, RNA therapy has flexibility and customizability, combining RNA therapy with targeted therapy. Its specific effect on the tumor microenvironment enhances therapeutic efficacy and reduces systemic side effects. However, RNA therapeutics confront several major challenges for wide clinical applicability, including large molecular weight, negative surface charge, and susceptibility to nuclease degradation, which demands more advanced RNA delivery vehicles with high efficacy and safety.

Extracellular vesicles (EVs) are natural membrane structures encapsulated by a lipid bilayer. Two subtypes of EVs, including EVs and microvesicles, are widely used for delivery.[6–12] EVs are formed through the inward budding of the plasma membrane, leading to the generation of early endosomes (ESE) and late endosomes (LSE). ESE and LSE subsequently develop into intracellular multivesicular bodies (MVBs) containing multiple intraluminal vesicles (ILVs).[13–16] Ultimately, ILVs are secreted as vesicles with a size ranging from 40 to 150 nm following the fusion of MVBs with the plasma membrane.[17–21] Microvesicles are larger vesicles, ranging from 50 to 1000 nm, and are generated by direct outward protrusion of the plasma membrane.[22,23] As an endogenous material, EVs possess advantages such as low immunogenicity and good biocompatibility.[24] They can be harnessed to deliver various therapeutic

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* Corresponding author.
** Corresponding author. School of Medical Engineering, School of Interdisciplinary Science, Affiliated Zhuhai People's Hospital, Beijing Institute of Technology, Zhuhai, 519088, PR China.
*** Corresponding author. School of Medical Engineering, School of Interdisciplinary Science, Affiliated Zhuhai People's Hospital, Beijing Institute of Technology, Zhuhai, 519088, PR China.
E-mail addresses: xinghn010@foxmail.com (H. Xing), yyhuang@bit.edu.cn (Y. Huang), lumei@bit.edu.cn (M. Lu).

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RNAs (e.g., cytokine mRNAs, tumor-associated antigen mRNAs, and siRNA therapeutics) (Fig. 1). The ability of EVs to freely circulate in the bloodstream, gain cellular entry, and deliver cargoes directly to the cytoplasm through membrane fusion enables them to address some disadvantageous properties of RNA molecules, such as degradation by endogenous nucleases, large-molecular weight, negative charge, and the entrapment in the endo-lysosomal compartments.[25] However, the targeting capacity of most natural EVs cannot meet the requirement of clinical utility. Modifications of EVs with tumor-targeting ligands may improve the delivery of therapeutic RNAs targeting specific tumor tissues or even tumor cells. The functionalization methods can be primarily categorized into three groups: (1) Genetic engineering of parental cells with targeting peptides or proteins to the scaffold proteins of EVs, such as lysosome-associated membrane glycoprotein 2 (Lamp2b); (2) Chemical modification of the surface proteins on EVs with targeting ligands, such as azide reactions[26]; (3) Formation of hybrid EVs through membrane fusion techniques. By utilizing the modified EVs to deliver therapeutic RNAs, it is possible to maximize the elimination of tumor cells while minimizing the impacts on healthy cells, thus providing a promising RNA delivery platform for targeted cancer treatment.

This review focuses on the potential of extracellular vesicles (EVs) as an RNA delivery platform for targeted cancer therapy. It discusses the challenges of RNA therapeutics, such as instability and poor delivery efficiency, and highlights the advantages of EVs in overcoming these barriers. The review covers various strategies for enhancing the targeting capacity of EVs, including genetic engineering of parental cells, chemical modifications, and hybrid EV formation. By exploring these strategies, the review underscores the potential of EVs to deliver therapeutic RNAs specifically to tumor cells, thereby maximizing therapeutic efficacy while minimizing side effects on healthy tissues. The paper ultimately presents EVs as a promising, innovative approach for overcoming current limitations in cancer treatment, with the goal of achieving more effective and personalized therapies.

2. Strategies for RNAs loading into EVs

2.1. Physical methods

Electroporation is the most used physical method for loading RNAs into EVs, which has been widely applied to load siRNAs, mRNAs, and other RNA molecules. During electroporation, electrical pulses are used to disrupt the EV membrane, allowing RNAs to enter the EV interior. Studies have shown that approximately 18 % of Cas9 mRNA can be loaded into red blood cell-derived EVs using electroporation .[27] In a recent study, Liu et al. used electroporation to load IL-12 mRNA into EVs, achieving an efficiency of 27.6 %.[28] The loading efficiency with electroporation typically ranges from 20 % to 30 %, with high efficiency for large RNA molecules (such as mRNAs). Other physical methods include sonication, extrusion, and freeze-thaw treatment. For example, sonication and extrusion methods have been used to load large proteins, such as catalase, into EVs.[29] However, these physical methods may compromise the integrity of EVs, potentially affecting their immunological properties.

2.2. Chemical transfection methods

Chemical transfection methods involve the use of transfection reagents (e.g., Exo-Fect, cationic liposomes) to encapsulate RNAs directly into EVs. These methods generally achieve high RNA loading efficiency and are particularly suitable for loading large RNA molecules. For instance, using the Exo-Fect transfection reagent, siRNAs targeting KRAS can be loaded into EVs with an efficiency of around 30 %.[30] However, these methods may introduce contamination from the transfection reagents, which can affect the subsequent applications of the EVs.

2.3. Engineered EVs strategies

Engineered EV strategies are crucial for enhancing RNA loading efficiency and functionality, employing cell engineering technologies and EV membrane modifications to improve EVs' ability to load specific RNA molecules and optimize their role as drug delivery carriers.

Firstly, surface fusion proteins and RNA binding proteins (RBPs) can be used to express specific fusion proteins on the surface of EVs, significantly improving RNA loading efficiency. Some RBPs (such as Male-specific lethal 2 and Poly(A)-binding protein, MSL2 and PABP) can specifically recognize and bind RNA molecules that contain packaging signals, which are typically composed of specific sequences or secondary structures that assist in RNA entry into EVs. By genetically engineering EVs source cells to overexpress these fusion proteins, RNA loading efficiency can be greatly enhanced, and RNA stability within EVs is improved, preventing degradation by intracellular enzymes.[31]

Additionally, self-assembled nanoparticles or vesicles present another effective engineered approach. Horns et al. developed a self-assembly RNA output system that incorporates protomers capable of assembling into dodecahedral nanocages, alongside RNA binding

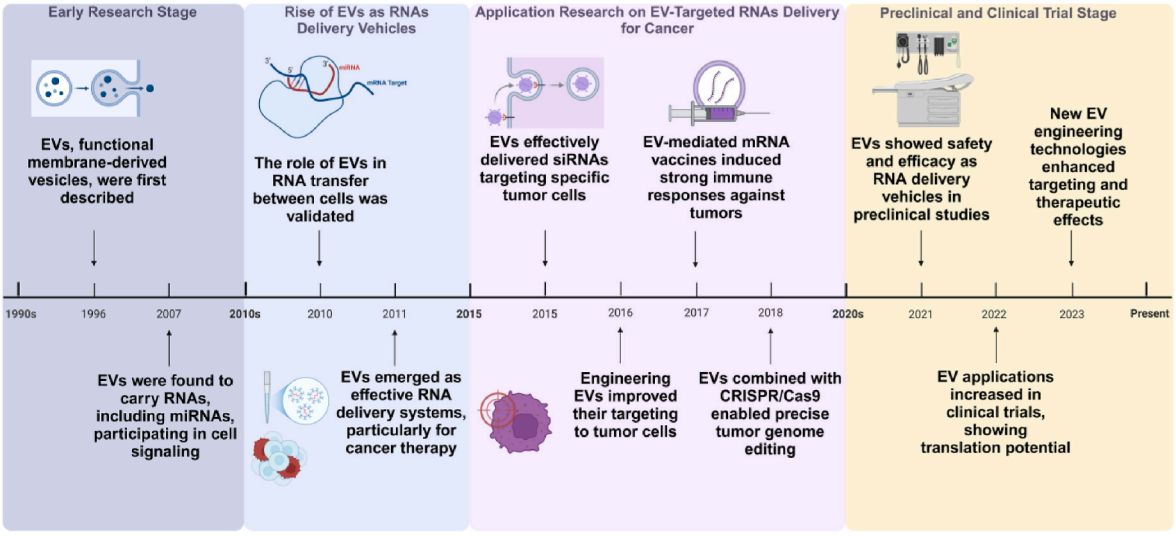

Fig. 1. Development of EV-encapsulated RNA therapies for cancer treatment.

proteins and membrane-binding domains.[32] This system facilitates the interaction between specific RNA molecules and self-assembling EV binding proteins, promoting efficient RNA entry into the EV lumen.[32] This method enables RNAs to be encapsulated and transported efficiently in a short time, while ensuring stability during transportation.

Membrane modification and the use of synthetic lipids are also common strategies. During EV membrane modification, synthetic lipids and chemically modified surface molecules are incorporated into the EV membrane to optimize RNA encapsulation capabilities. These modifications can enhance the fluidity and stability of the EV membrane, increasing its ability to accommodate RNAs and improve EVs' membrane penetration ability. Altering the lipid composition of the EV membrane also allows for the regulation of EV size, morphology, and surface charge, further enhancing their targeting ability and drug delivery effectiveness.[33] Additionally, specific chemical linkages can be used to attach target RNA molecules to ensure effective encapsulation within EVs.

These engineered strategies significantly enhance EVs' RNA loading capacity, stability, and targeting capabilities, improving their functionality as drug delivery vehicles and advancing applications in gene therapy, vaccine development, and other fields.

3. Strategies for engineering EV-based tumor-targeted therapy

EVs represent a promising drug delivery vehicle with a suitable size and carrying some specific components, which may endow them with tumor accumulation features.[34] EVs from specific cell types, such as those derived from natural killer cells (NKEXOs), exhibit inherent specificity towards tumor cells.[35] However, the majority of natural EVs exhibit limited tumor-targeting characteristics, thereby necessitating modifications to enhance their tumor-targeting capabilities. Chemical modifications of EVs or membrane fusion techniques have been extensively developed to enhance the tumor targeting of EVs. Compared to artificially synthesized nanoparticles (e.g., lipid nanoparticles, LNPs), EVs can be genetically engineered for surface modifications, which allows for continuous production of tumor-targeting EVs.[25,36] For instance, a landmark study by Matthew et al. at Oxford University demonstrated that dendritic cell-derived EVs (DEX) expressed RVG-targeting peptides by using genetic fusion. RVG-modified DEX effectively delivered siRNAs across the blood-brain barrier (BBB) targeting the brain.[37] In this section, the tumor-targeted strategies for EVs will be elaborated (Fig. 2).

3.1. Ligand-based targeting strategies

By expressing targeting ligands on the surface of EVs, it is possible to direct them towards tumor cells. The types of ligands typically include targeting peptides, antibodies, and aptamers. Targeting peptides are one of the most commonly used targeting ligands due to their low immunogenicity, small molecular weight, and strong penetrative ability. The most common expression method involves fusing ligands to Lamp2b through genetic engineering. For example, the RVG targeting peptide can be fused with Lamp2b to target the brain[37,38] Immature dendritic cells (imDCs) expressing iRGD-Lamp2b fusion proteins can produce EVs loaded with the chemotherapeutic drug doxorubicin (DOX) for targeted delivery to breast cancer cells.[39] In addition to peptides, antibodies have also been used for targeted delivery by fusing with phosphatidylserine (PS)-binding domains of lactadherin (C1C2). Kooijmans et al. demonstrated that expressing a fusion protein of anti-epidermal growth factor receptor (EGFR) nanobody (EGa1-C1C2) could achieve high affinity binding to EGFR.[40] Similarly, Longatti et al. expressed anti-HER2 (Human epidermal growth factor receptor 2) single-chain variable fragments to target HER2-positive tumor cells.[41] In another study, anti-EGFR camelid biparatopic nanobodies (variable homodimers, VHH) were utilized to target EGFR-positive lung cancer cells. The targeting EVs achieved effective treatment of lung cancer in mice after being loaded with paclitaxel.[42]

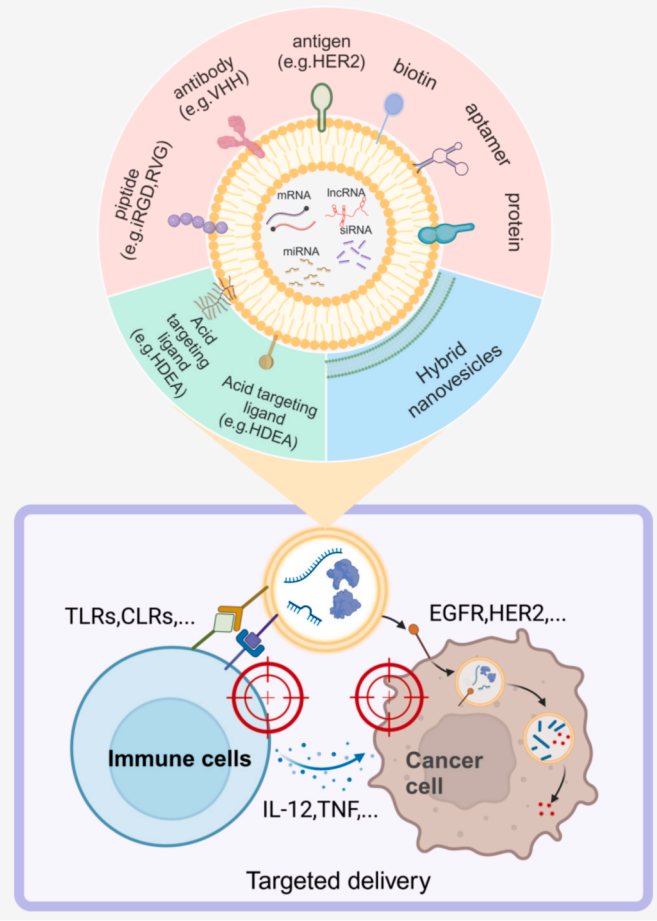

Fig. 2. Strategies for engineering EVs to deliver RNAs for targeted tumor therapy. The strategies for engineering EVs to deliver RNA molecules specifically for targeted tumor therapy. By modifying EVs for enhanced stability and targeting, they can effectively deliver therapeutic RNAs, enabling targeted cancer treatments while minimizing off-target effects.

Besides genetic engineering of parental cells, non-genetic modification methods can be employed for EV modification.[43] For example, Jia et al. designed engineered EVs loaded with superparamagnetic iron oxide nanoparticles (SPIONs) and curcumin (Cur), and chemically modified EV surfaces with neuropilin-1-targeted peptides (RGERPPR, RGE).[44] RGE effectively guided EVs across the BBB to target gliomas. Moreover, SPION-induced magnetic flow hyperthermia (MFH) synergistically helped to kill tumors.[44] In another study, Wang et al. anchored biotin to the donor cell membrane via 1, 2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-polyethylene glycol (DSPE--PEG), and then used streptavidin to bind biotin to functionalize EVs with dual ligands targeting biotin receptors and lectins overexpressed in tumor cells.[45] By using a palmitic acid tail, Dusoswa et al. anchored a ligand Lewis^{Y}, which specifically bound to dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN, CD209), on the surface of EVs derived from glioblastoma to prepare multi-antigen pulsed tumor vaccines.[46] Likewise, diacyl lipid tails were used in another study to display aptamers on the EV surface by using the hydrophobic interactions between the lipid and the phospholipid bilayer of cells to achieve tumor-targeted therapy.[47]

3.2. Stimuli-responsive EV-based targeting strategies

In addition to active targeting via ligand-receptor interactions, EVs can also be modified to target specific cells or tissues by taking

advantage of the specific properties of the tumor microenvironment, such as acid and enzymes. To be exemplified, modification of EVs with hyaluronic acid grafted with 3-(diethylamino) propylamine (HDEA) enabled them to target the acidic tumor microenvironment (pH 6.5) and bind with high affinity to CD44 receptors on colorectal cancer cells.[48] The double-stranded biotin-i-motif (ds-i-motif-bio) can be used to modify EVs in response to acidic pH environments through biotin-streptavidin interactions.[49] In 2021, Gong et al. incorporated photosensitizers into low pH-reprogrammed EVs, allowing them to target tumors. Upon reaching the tumor site, near-infrared laser irradiation induced the release of the loaded chemotherapeutic agents at the tumor site,[50] and significantly enhanced the therapeutic efficacy while reducing damage to healthy cells.

By adding magnetic particles to the surface of EVs, external magnetic field stimulation can also be used to enhance the targeting ability of EVs. SPIONs exhibit magnetic properties under alternating magnetic fields and can be directed accordingly, making them frequently applicable in targeted therapies.[51] In one study, superparamagnetic magnetite colloidal nanocrystal clusters (SMCNCs) were used to modify reticulocyte (RTC)-derived EVs. By using moderate magnetic fields (MFs), the modified EVs enabled the control of their movement in the bloodstream to target tumors.[52] Zhuang et al. utilized genetic engineering to express cell-penetrating peptides (CPP) and a TNF-α (CTNF-α) fusion protein in mesenchymal stem cells (MSCs), anchoring TNF-α to the EV membrane. They then added SPIONs to the EVs through transferrin–transferrin receptor (Tf-TfR) interactions to achieve tumor targeting.[53] Similarly, a recent study loaded SPIONs in neutrophil-derived EVs (N-EX) to target human gastric cancer cells (HGC27). The targeting EVs were then used to load DOX to achieve favorable tumor treatment outcomes. Compared to the liposome-loaded DOX and naturally derived neutrophil nanovesicles (NNV), the engineered EVs exhibited superior anti-tumor activity.[54]

3.3. Hybrid nanovesicle-based targeting strategies

Leveraging the specificity of certain natural EVs towards tumors and the homing ability of tumor cell-derived EVs, hybridization of tumor cell and immune cell-derived EVs can be achieved. This can be accomplished through membrane fusion between EVs or by fusing artificially synthesized nanoparticles (such as LNPs) with the EV membrane. In one study, macrophages were utilized to internalize the nuclei of tumor cells, and hybrid EVs from tumor cells and M1 macrophages were generated. These hybrid EVs exhibited enhanced homing capabilities, thus accumulating in tumor sites and lymph nodes. As a result, the engineered EVs achieved improved tumor suppression and activated immune cell-mediated tumor cytotoxicity.[55] To validate the applicability of this method across various tumor models, the team incubated the nuclei of lymphoma cells, breast cancer cells, and melanoma cells with macrophages, and subsequently treated these three tumor models with the obtained EVs. All the engineered EVs demonstrated significant anti-tumor efficacy. In another similar study, Bao et al. hybridized dendritic cells (DCs) with tumor cells, incorporating STING activation molecules into the chimeric EVs produced by electroporating hybrid cells. This approach not only enhanced the homing ability of EVs to cross the blood-brain barrier (BBB) and target the glioblastoma tissue but also effectively activated immune cells and reversed the immunosuppressive microenvironment of glioblastoma.[56]

Chimeric antigen receptor (CAR)-T cell therapy has shown promising results in clinical studies for targeting tumors, however, it is associated with obvious cytotoxicity.[57] In one study, 23–46 % of patients with acute lymphoblastic leukemia/lymphoma (ALL/LBL) exhibited systemic cytokine release and extensive T-cell expansion.[58] Conversely, CAR-T cell-derived EVs inherited the tumor-targeting properties of CAR-T cells while exhibiting lower toxicity and higher safety. Moreover, EVs are small-sized, therefore, they can accumulate in tumor sites more efficiently following administration.[59] Additionally, this research also confirmed the efficacy of CAR EVs in xenograft tumor models of human non-small cell lung cancer and human breast cancer. Moreover, while CAR-T cells may become inactive due to pathways involving programmed cell death ligand 1 (PD-L1), CAR EVs do not exhibit this limitation.[59] Zhu et al. fused mesothelin (MSLN) and PD-L1 bispecific antibodies to EVs derived from donor cells and combined them with lung-targeting liposomes for the delivery of the chemotherapeutic drug paclitaxel (PTX). This dual-targeting approach effectively targeted MSLN-positive tumor cells and the lungs, thereby avoiding the systemic side effects of PTX while enhancing its delivery to tumor sites. Furthermore, the PD-L1 antibodies on the surface of CAR EVs can block PD-L1 on tumor cells, and the granzyme B and perforins contained within EVs can enhance tumor cytotoxicity. This system achieved precise drug delivery in a CT26 metastatic lung cancer model, which resulted in excellent antitumor efficacy and prolonged survival of tumor-bearing mice.[60] Dual-targeting systems integrating bispecific antibodies and chemotherapeutic drugs further enhance therapeutic efficacy by precisely delivering agents to tumor cells and blocking immune evasion mechanisms such as PD-L1 expression. These advanced EV-based strategies showcase the immense potential of combining natural and engineered approaches for robust and efficient cancer treatments.

In summary, targeting peptides (such as RVG and iRGD) have demonstrated significant tumor specificity, particularly in glioma and breast cancer models, exhibiting low immunogenicity and strong penetrative ability,[37–39] although their targeting efficiency is generally lower compared to other methods such as antibodies. Targeting antibodies (such as anti-EGFR and anti-HER2 nanobodies) have been effectively used to direct EVs to specific tumor cells, with high binding affinity and targeting precision, as evidenced by studies involving lung cancer and HER2-positive tumors, but the penetrative ability may be limited [40] 27]. Aptamers extend the multifunctionality of EV targeting by showing a strong affinity for specific tumor markers and enabling efficient therapeutic delivery, offering a middle ground in terms of specificity and penetrative ability. Stimuli-responsive engineered EVs, such as those loaded with HDEA or SPIONs, can also enhance targeting capabilities, enabling precise tumor localization through tumor microenvironmental properties or magnetic manipulation, providing a higher degree of tumor localization than peptides or antibodies in models like glioma and cancer[48,54]. However, the targeting efficiency can be lower compared to hybrid systems. Hybrid systems, combining tumor cell-derived EVs with immune cell-derived EVs or nanoparticles, provide enhanced homing abilities and tumor suppression, showing promise in overcoming the limitations of single-target approaches. For instance, hybrid EVs generated by fusing tumor cell and macrophage membranes showed improved accumulation in tumors and lymph nodes, outperforming both peptides and antibodies in terms of homing ability, while enhancing immune cell-mediated tumor cytotoxicity.[45] Furthermore, CAR-T cell-derived EVs have emerged as a safer alternative to traditional CAR-T cell therapy, maintaining tumor-targeting properties while reducing systemic toxicity. When combined with bispecific antibodies and chemotherapeutic drugs, these dual-targeting systems significantly improved therapeutic outcomes, enabling efficient drug delivery and overcoming immune evasion mechanisms such as PD-L1 expression.[60] Dual-targeting systems, therefore, exhibit higher therapeutic potential than single-target strategies, offering better tumor suppression and reducing toxicity. Overall, while each modification strategy offers distinct advantages in terms of specificity and therapeutic potential, hybrid and dual-targeting systems are emerging as particularly promising approaches, combining the strengths of multiple strategies to enhance targeting efficacy, reduce toxicity, and improve tumor suppression. These advancements highlight the immense potential of engineered EVs in precision cancer therapy.

4. EVs as Tumor-Targeting Nanocarriers for mRNA delivery

mRNAs are easily produced and can be designed and manufactured rapidly for various therapeutic applications. They are relatively safe and can provide long-lasting therapeutic effects, thus showing significant potential in cancer treatment in recent years. Additionally, mRNAs can be engineered to encode specific proteins that help target and treat cancer cells more precisely. They do not require the use of live viruses for delivery, reducing the risk of adverse immune reactions. Moreover, mRNA-based therapies offer flexibility, as they can be quickly adapted to respond to new cancer types or emerging mutations, making them a versatile and adaptive option in personalized medicine (Table 1). mRNA's ability to elicit both humoral and cellular immune responses further enhances its efficacy in cancer immunotherapy.[61] However, mRNAs are highly prone to degradation and face significant challenges in achieving efficient cellular uptake. To address these limitations, recent studies have explored the application of LNPs, polymeric nanoparticles (PNPs), and inorganic nanoparticles as nanocarriers for mRNA delivery. These nanocarriers exhibit stable nanostructures, enabling fusion with negatively charged endosomal membranes and facilitating effective nucleic acid delivery. Additionally, they can be precisely engineered to achieve optimal sizes for cellular uptake. Despite these advancements, the cytotoxic effects induced by these carriers and their ability to trigger innate immune responses remain considerable

Table 1
Strategies for targeted delivery of RNAs to tumors.

Type of RNAs	Therapeutic RNAs	Tumors	Targeted strategies	Outcomes	References
mRNAs	HChrR6 mRNA	Breast Cancer	EVHB chimeric protein	The prodrug CNOB is converted to the cytotoxic drug MCHB by HChrR6, and the tumor is inhibited	63,64
	PTEN mRNA	Glioblastoma	CDX/CREKA peptide	Prolonging circulation half-life and showing high safety in tumor-bearing mice	65
	IFNγ mRNA	Glioblastoma	anti-CD71 and anti-PDL1 antibodies	Activating T cells and inhibiting tumor growth	66
miRNAs	let-7a miRNA	Breast Cancer	GE11 peptide	Inhibiting tumor growth	67
	let-7a miRNA	Glioblastoma	NKEXOs in combination with biomimetic core-shell nanoparticles	Natural NKexo accumulates specifically in the tumor tissue, and the tumor is inhibited	68
	miR-26a	Liver Cancer	Apo-A1	Inhibiting tumor growth	69
	miR159	Breast Cancer	a disintegrin and metalloproteinase 15 (A15)	Inhibiting tumor growth	70
	miRNA-126	Non-Small Cell Lung Cancer	integrin β_4	Effectively interrupting the PTEN/PI3K/AKT signaling pathway and inhibiting tumor growth	71
	miR497	Ovarian cancer	Hybrid nanoparticles of cRGD-modified liposomes and TEX	Polarization of macrophages from M2 to M1 and inhibition of tumor growth	72
siRNAs	PD-L1 siRNA	Colorectal Cancer	Natural homing characteristics of M1 EVs	Blockade of the PD-L1/PD-1 interaction and inhibition of tumor growth	73
	Galectin-9 siRNA	Pancreatic cancer	Natural homing characteristics of BM-MSC EVs	Inhibiting tumor growth	74
	CDK1 siRNA	Liver Cancer	Natural homing characteristics of TEX	Gene silencing and inhibition of tumor growth	75
	siS100A4	Breast Cancer	Natural homing characteristics of TEX	Inhibiting tumor growth and metastasis	76
	CPT1A siRNA	Colorectal Cancer	iRGD	Overcoming antitumor drug resistance and inhibiting tumor growth	77
	TPD52 siRNA	Breast Cancer	DARPin G3	Downregulating of TPD52 and inhibiting tumor growth	78
	KRAS siRNA	Lung Cancer	iRGD	Inhibiting tumor growth	79
	KRAS siRNA	Lung Cancer	FA	Inhibiting tumor growth	80
	SOX2 siRNA	Non-Small Cell Lung Cancer	tLyp-1	Inhibiting tumor growth	81
	SIRT6 siRNA	prostate cancer	Aptamers	Established the underlying mechanism of SIRT 6 activation in various cancer-associated signaling pathways, and demonstrated favorable therapeutic efficacy for prostate cancer	82
	FGL1 and TGF-β1 siRNA	Colorectal Cancer	cRGD	Inhibiting tumor growth	83
	Bcl-2 siRNA	Breast Cancer/Tongue Squamous Cell Carcinoma	FA	Enhancing tumor cell toxicity by 15 %	84
	Survivin siRNA	Prostate/Breast/ Colorectal cancer	PSMA/EGFR/FA	Downregulating the survivin gene and inhibiting tumor growth	85,86
	EGFR and KRAS siRNA/EGFR and TNC siRNA	Lung Cancer/ Glioblastoma	peptides	Inhibiting tumor growth	87
	Bcr-Abl siRNA	Chronic Myeloid Leukemia	IL3	Inhibiting tumor growth	88
Other RNAs	STAT6 ASO	Colorectal Cancer /Hepatocellular Carcinoma		Polarization of macrophages and inhibition of tumor growth	89
	miRNA-221 AMO	Colorectal Cancer	iRGD	Inhibite tumor growth	90
	miRNA-21 AMO	Glioblastoma	T7	Inhibiting tumor growth	91
	miRNA-21 AMO	Colorectal Cancer	Her2	Enhancing the cytotoxicity of 5-FU and inhibiting tumor growth	92
	RN7SL1	Lung Cancer	CAR-T EVs	Enhancing the presentation of tumor cell antigens, activating myeloid cells and dendritic cells, and hence initiatting T cell immune rejection of tumors that had lost CAR antigens	93

challenges.[62] A comprehensive understanding of the mechanisms underlying carrier-mediated mRNA delivery, coupled with innovative strategies to mitigate these adverse effects, will be critical in advancing these systems toward clinical translation. This underscores the necessity of developing next-generation nanocarriers with enhanced biocompatibility and improved delivery efficacy.

In contrast, harnessing EVs for targeted delivery of mRNAs may offer higher safety and lower immunogenicity. Due to their rich composition of sphingolipids, cholesterol, and phospholipids, they can achieve a higher degree of complexity. The use of EVs for mRNA-targeted cancer therapy has been extensively studied (Fig. 3). For instance, Wang et al. utilized EVHB proteins, which contain anti-HER2 scFv antibodies expressed on the EV membrane and can target $HER2^{+}$ tumor cells, to modify EVs and loaded them with the bacterial enzyme HChrR6 mRNA21. Following administration, the prodrug CNOB is converted by HChrR6 into the cytotoxic agent MCHB, thus contributing to effective tumor-killing activity and nearly complete tumor regression in tumor-bearing mice.[63] This study marked the first use of EVs as a tumor-targeted delivery nanoplatforms for therapeutic mRNAs and paved the way for mRNA-based cancer therapies. In a subsequent study, the team further demonstrated that EVs could also deliver HChrR6 mRNA that was transcribed *in vitro*, and achieved similar cytotoxic effects against $HER2^{+}$ tumor cells BT47E4.[64]

However, the large molecular weight of mRNAs has historically posed challenges for their loading into EVs. To enhance loading efficiency, Yang et al. developed a nanopore electroporation technique that increased EV yield by 50-fold and allowed for over a 103-fold increase in mRNA encapsulation within EVs. Using this technique, therapeutic mRNA PTEN was loaded in EVs that were modified with a glioma-targeting peptide by inserting at the N-terminus of the CD47 transmembrane protein expressed on the EV surface. As a consequence, the engineered EVs endowed effective delivery of PTEN mRNA across the BBB for glioma treatment.[65] This system not only improved EV yield but also significantly enhanced the loading capacity of therapeutic mRNAs within EVs. Furthermore, the engineered EVs ultimately contributed to prolonged circulation half-life and high safety in tumor-bearing mice, thus opening a new avenue for targeted mRNA delivery using EVs. Similarly, Dong et al. employed a nanosecond pulsed electroporation method to load IFNγ mRNA into EVs, which were engineered to stably express CD64. CD64 serves as a docking site for anti-CD71 and anti-PD-L1 antibodies that allow the resulting EVs to target glioblastoma cells. IFNγ upregulated the expression of major histocompatibility complex class I (MHC-I) and helped to activate T cells, which ultimately led to tumor cell death.[66] Additionally, the team designed an active loading mechanism for IFNγ mRNA by cloning an N-peptide to the C-terminus of the CD64 protein and box B sequence into the engineered IFN-γ plasmid (Fig. 4D). This clever design allowed for the specific binding of IFN-γ mRNA to EVs, with the N-terminus docking site positioned on the surface to facilitate targeting of tumor cells.[66] This study integrates nanosecond pulsed electroporation technology, engineered CD64 protein design, immune function enhancement, and specific mRNA active loading strategies to achieve efficient and precise delivery of mRNAs and proteins. These innovations significantly improve the loading capacity and targeting capabilities of EVs, addressing the challenge of low mRNA loading efficiency. By leveraging a transmembrane protein to actively recruit target mRNAs and incorporating interface protein CD64 on the vesicle surface for antibody presentation, this approach introduces a novel strategy for tumor immunotherapy with broad translational potential.

5. EVs as Tumor-Targeting Nanocarriers for miRNA delivery

miRNAs are another type of RNA molecule that can regulate the stability of mRNAs through recognition sites at the 3′ untranslated region (UTR),[94] thereby influencing gene expression. One key advantage of miRNAs is their ability to regulate multiple target genes simultaneously, which can lead to coordinated modulation of complex cellular pathways, offering potential therapeutic benefits in diseases like cancer and cardiovascular disorders (Table 1). Additionally, Let-7a miRNA inhibits tumor cell proliferation by downregulating the expression of RAS and HMGA2, functioning as a widely recognized tumor suppressor. In one study, the expression of GE11 (amino-acid sequence YHWYGYTPQNVI) peptide on the surface of EVs enabled targeted delivery of let-7a miRNA to EGFR-positive breast cancer cells for tumor treatment.[67] This approach highlights the potential of engineered EVs in enhancing the specificity and efficacy of miRNA-based therapies. Another study employed a “cocktail therapy” approach for targeted delivery of let-7a miRNA. Specifically, tyrosine-coupled dendrimers loaded with let-7a miRNA were encapsulated in EVs to form biomimetic core-shell nanoparticles (NNs), leveraging the natural hydrophobic properties of the lipid bilayer in EVs. As a consequence, this therapy achieved good therapeutic effects in a mouse glioblastoma model due to the specific accumulation of natural NKEXOs in tumor tissues without affecting normal cells.[68] Together, these studies demonstrate the

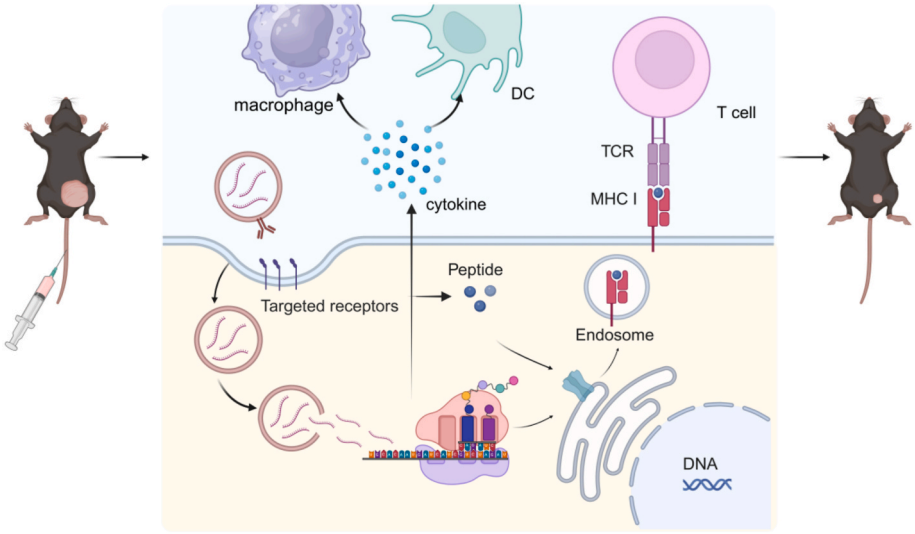

Fig. 3. Engineering EVs for mRNA Delivery to Enhance Antitumor Immunity. EVs loaded with mRNA-encoding cytokines or tumor antigens are designed to target tumor sites. Upon delivery, the mRNA is translated, producing functional proteins that activate macrophages, DCs, or T cells. This activation enhances antitumor immunity, promoting tumor cell recognition and eradication.

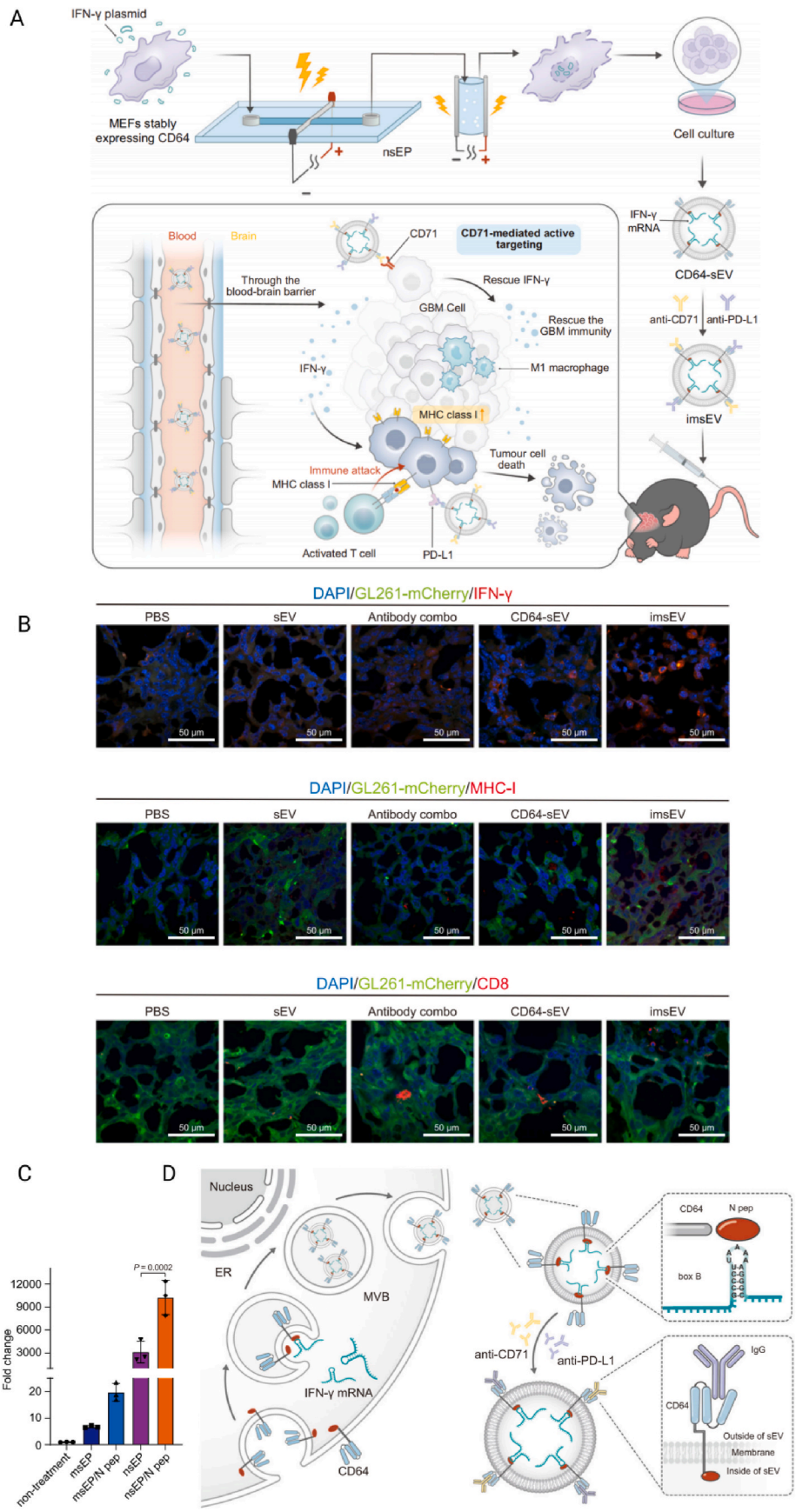

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Fig. 4. EVs as Tumor-Targeting Nanocarriers for IFNγmRNA Delivery. (A) Schematic overview of the large-scale production process and therapeutic action of EVs. (B) Staining of tumor tissues in the treatment group showed that EVs increased IFN-γ, MHC-I expression, and the infiltration of $CD8^+$ T cells. (C) Quantification of IFNγ in EVs. (D) The diagram illustrated the functional integration of IgG onto the surface of EVs through CD64 and the RNA packaging system utilizing the N peptide-box B interaction. Adapted with permission from Dooley et al.[56] Copyright 2023, Springer Nature.

versatility of EVs in delivering miRNAs to tumor cells, providing a promising avenue for future cancer treatments.

miR-26a is another popular miRNA for cancer treatment. It is downregulated in liver cancer cells, and systemic administration of miR-26a in liver cancer mice can induce tumor-specific apoptosis.[95] This demonstrates the therapeutic potential of miR-26a in targeting liver cancer through apoptosis induction. Liang et al. engineered a fusion protein comprising the EV membrane protein CD63 and apolipoprotein A-I (Apo-A1) to enhance EV targeting specificity toward HepG2 cells. Apo-A1, the primary protein component of high-density lipoproteins (HDL), plays a critical role in reverse cholesterol transport by mediating cholesterol efflux from peripheral tissues to the liver for excretion. Additionally, Apo-A1 exhibits anti-inflammatory, antioxidant, and cardioprotective properties. By leveraging these natural biological functions, the fusion of Apo-A1 with CD63 facilitated the precise delivery of EVs to HepG2 cells, highlighting a novel strategy for tumor-specific targeting in liver cancer models. Subsequent electroporation loading of miR-26a resulted in the growth inhibition of tumor cells.[69] Recent studies have explored new targeting peptides, such as a disintegrin and metalloproteinase 15 (A15), which contains the Arg-Gly-Asp (RGD) motif that binds to integrin αvβ3 in an RGD-dependent manner. The use of such targeting peptides represents an innovative strategy to improve EV-mediated miRNA delivery by leveraging tumor-specific surface markers. The expression of A15 on the EV surface enabled them to target different kinds of tumors overexpressing integrin αvβ3, including melanoma, glioma, and breast cancer, and enhanced their cellular uptake in tumor cells.[96] Existing research indicates that the level of miRNA 159 (miR159) is negatively correlated with the occurrence and progression of breast cancer.[97] Gong et al. prepared A15-modified EVs to co-deliver miR159 and doxorubicin and achieved potent antitumor effects against triple-negative breast cancer.[70] These studies underline the versatility of A15-modified EVs in targeting multiple tumor types and enhancing therapeutic efficacy. Additionally, research has shown that the surfactant protein C (SPC) expressed in A549 non-small cell lung cancer cells can specifically bind to integrin β4 on the surface of EVs derived from breast cancer cells. Loading miRNA-126 into the engineered EVs targeting A549 cells effectively interrupted the PTEN/PI3K/AKT signaling pathway, resulting in significant inhibition of tumor growth and metastasis.[71] This mechanism not only underscores the role of miRNA-126 in modulating critical cancer pathways but also highlights the specificity of EV-based targeting strategies in complex tumor microenvironments.

Also, hybrid nanoparticles can be developed for targeted delivery of miRNAs in cancer therapy. For example, Li et al. modified LNPs with triptolide (TP) and cRGD, which were then fused with tumor-derived EVs expressing CD47 to generate hybrid nanoparticles. These nanoparticles adsorbed miR497 on their surface through calcium phosphate (CaP) (Fig. 5A). The results indicated that these nanoparticles could effectively accumulate in tumor regions, resulting in the dephosphorylation of the overactive PI3K/AKT/mTOR signaling pathway, generation of reactive oxygen species (ROS), and polarization of macrophages from M2 to M1. These effects collectively contributed to significant tumor suppression[72](Fig. 5B–D). The innovation of this study lies in the combination of tumor EVs with functional nanoparticles, leveraging the tumor-targeting ability of EVs and the drug delivery capacity of nanoparticles, addressing the issues of poor targeting and high side effects associated with traditional therapies. Furthermore, by modulating the PI3K/AKT/mTOR signaling pathway and macrophage polarization, this study provides new insights into the intervention of the tumor microenvironment, demonstrating the potential of nanomedicine in cancer treatment.

6. EVs as Tumor-Targeting Nanocarriers for siRNA delivery

Unlike endogenous miRNAs, small interfering RNAs (siRNAs) are generally used as exogenous RNA molecules. The mechanism of action is that siRNAs can bind to the RNAs-induced silencing complex (RISC), which subsequently binds to target mRNAs, thus leading to its degradation and thereby gene silencing.[98,99] The advantages of siRNAs include their high specificity in targeting and silencing individual genes, which allows for precise regulation of gene expression. This precision is particularly valuable in treating genetic diseases, viral infections, and certain cancers. Moreover, siRNAs can be designed to target a wide range of disease-associated genes, and their efficacy is enhanced when delivered using advanced delivery methods, such as exosome-based systems (Table 1). The targeted delivery of siRNAs to tumor tissues can leverage the homing properties of certain EVs. For instance, Liu et al. utilized a pH-responsive viral fusion protein, vesicular stomatitis virus glycoprotein (VSV-G), to modify M1 macrophage-derived EVs (M1EVs) and then loaded EVs with anti-PD-L1 siRNA.[73] By combining the natural tumor-targeting capacity of M1EVs with the membrane fusion ability of VSV-G, these engineered EVs facilitated direct cytoplasmic delivery of siRNAs, leading to robust gene silencing, effective blockade of the PD-L1/PD-1 interaction, and significant antitumor effects. Similarly, Zhou et al. exploited the tumor-homing characteristics of bone marrow-derived mesenchymal stem cell (BM-MSC) EVs by surface-modifying the prodrug oxaliplatin (OXA) and loading it with galectin-9 siRNA.[74] This dual approach not only improved the tumor microenvironment but also activated immune cells, triggering potent antitumor immunity and demonstrating substantial therapeutic efficacy in cancer treatment. Together, these studies underscore the transformative potential of EV-based delivery systems for siRNA therapies, highlighting their ability to integrate precise tumor targeting with multifunctional therapeutic strategies.

By fusing lipid nanoparticles with EVs to create hybrid nanovesicles, it is possible to retain the targeting capabilities of EVs while substantially increasing EV production. The extrusion of lipid formulations (DOTAP, POPC, DPPC, and POPG) with EVs resulted in a 6-to-43-fold increase in the number of isolated vesicles, with a 14-enhancement in uptake by A549 lung cancer cells. Electroporation of siRNAs in the hybrid EVs achieved a gene-silencing effect comparable to that of the commercial lipid-based formulation RNAsiMax.[100] Similarly, Zhou et al. created hybrid nanovesicles by fusing EVs derived from liver cancer cells with phospholipids, which exhibited targeting capabilities toward the parent liver cancer cells. This approach facilitated a 1. 7-fold increase in the transfection efficiency of loaded CDK1 siRNA, thus resulting in effective gene silencing and significant anti-tumor efficacy.[75] Zhao et al. purified EVs with the lung-targeting capability from breast cancer cells and used these EVs to encapsulate cationic bovine serum albumin (CBSA) conjugated with siS100A4. The resulting hybrid nanovesicles demonstrated targeted delivery to the lungs and exhibited significant gene-silencing effects, concurrently inhibiting the growth and metastasis of breast cancer cells.[76]

Genetically engineered EVs have also been extensively developed for tumor-targeted siRNA delivery. For example, Lin et al. developed iRGD-modified EVs loaded with carnitine palmitoyltransferase 1A (CPT1A) siRNA, a key enzyme in fatty acid oxidation (FAO) that promoted drug resistance in cancer cells. This system effectively suppressed CPT1A expression and significantly inhibited tumor cell growth when combined with OXA.[77] Similarly, Limoni et al. modified the surfaces of EVs with

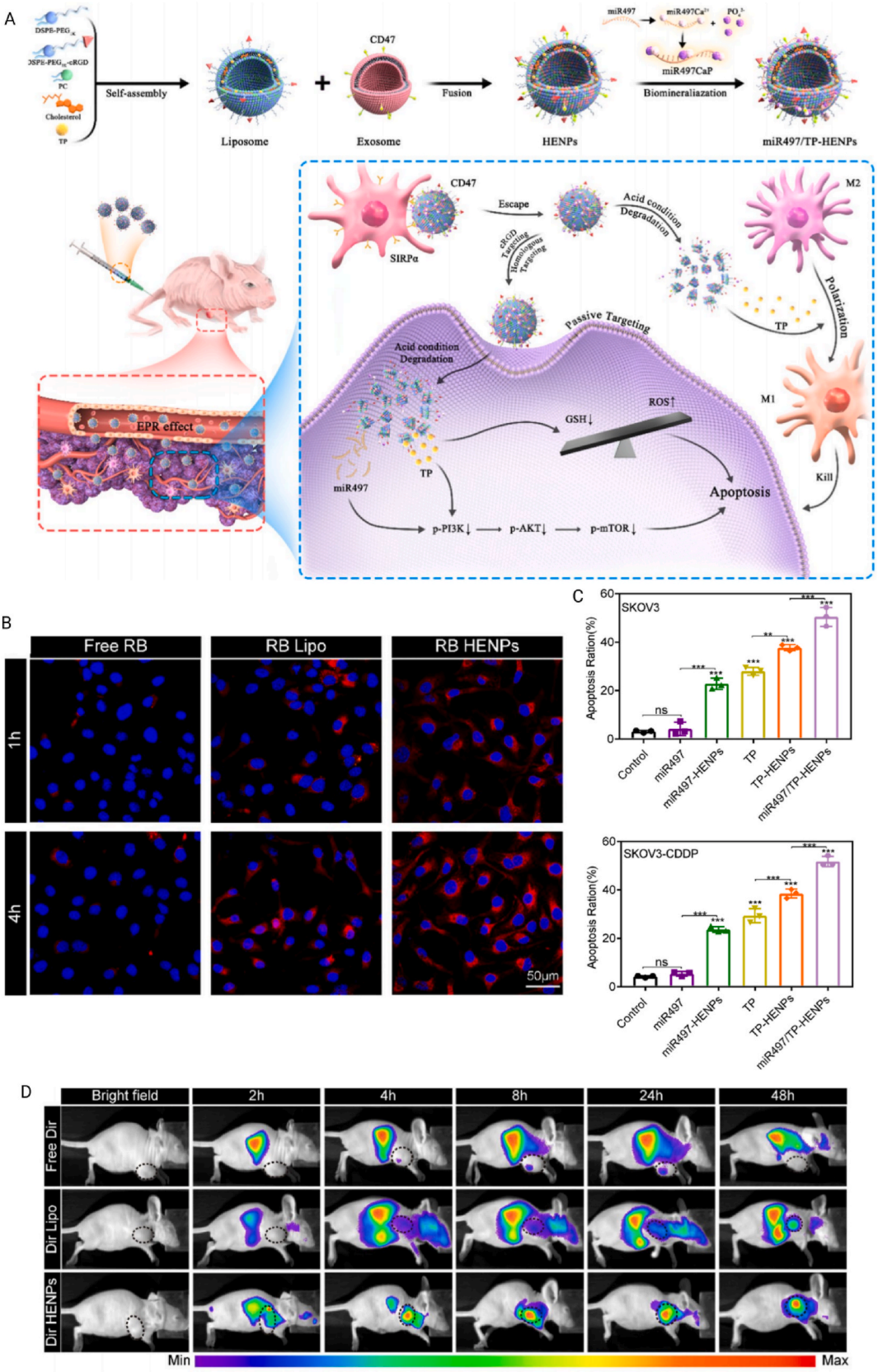

Fig. 5. EVs as Tumor-Targeting Nanocarriers for mi497 Delivery. (A) Tumor-derived EVs fused with LNPs loaded with mi497 promoted M1 polarization and tumor cell killing. (B) Hybrid nanoparticles enhanced the uptake by tumor cells. (C) Hybrid nanoparticles promoted tumor apoptosis. (D) Hybrid nanoparticles exhibited *in vivo* tumor-targeting ability. $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data in (C) are presented as mean ± standard deviation (SD). (A)–(D) adapted with permission from Li et al.[66] Copyright 2022, Springer Nature.

DARPin G3 to achieve specific binding to HER2/Neu, resulting in a 70 % downregulation of TPD52 gene expression through the delivery of corresponding siRNAs.[78] Zhou et al. engineered EVs expressing iRGD-Lamp2b fusions to deliver KRAS siRNA for targeted therapy in lung cancer.[79] Munagala et al. utilized bovine colostrum EVs and a polyethyleneimine matrix to deliver KRAS siRNA, which effectively inhibited lung cancer cell growth.[80] Han et al. employed aptamer-modified EVs to deliver SIRT6 siRNA investigated the mechanisms of SIRT6 activation in various cancer-related signaling pathways and demonstrated promising therapeutic effects in prostate cancer.[82] Pei et al. developed cRGD-modified EVs co-loaded with siFGL1 and siTGF-β1, which exhibited significant anti-tumor effects, both *in vitro* and *in vivo*.[83]

Another subtype of EVs, microvesicles (MVs), are less commonly used as nanocarriers due to their larger size. However, Zhu et al. utilized MVs as siRNA delivery vehicles, employing biotin and folate modifications to load Bcl-2 siRNA and paclitaxel. The engineered MVs exhibited high targeting capabilities towards tumor tissues and demonstrated synergistic anti-tumor effects, ultimately enhancing tumor cytotoxicity by 15 %.[84]

The aforementioned methods primarily involve genetic engineering to modify the surfaces of EVs with targeting ligands. To develop a more versatile engineered EV delivery vehicle, Pi et al. utilized RNA nanotechnology for directional control, altering the orientation of antibody-like (arrow-shaped) RNAs to display ligands on EV membranes for tumor cell targeting. By placing membrane-anchored cholesterol at the tail of the arrow, RNA aptamers or folate could be displayed on the surface of EVs.[85] Conversely, placing cholesterol at the arrowhead facilitated RNA loading into EVs. Ligands corresponding to prostate-specific membrane antigen (PSMA), EGFR, and folate receptors were added to achieve targeted therapy in prostate cancer, breast cancer, and colorectal cancer models, respectively. Furthermore, all EVs were loaded with siRNAs to downregulate the survivin gene, which effectively inhibited tumor growth.[85] To explore the exact mechanisms of folate-EV complexes, the authors demonstrated that folate-modified EVs could achieve endosomal escape and efficiently deliver siRNAs to the cytoplasm. Compared to unencapsulated FA-siRNA, these modified EVs exhibited significant gene knockout effects both *in vitro* and *in vivo*, which supported the excellent therapeutic outcomes observed.[86] In addition, Fu et al. pioneered a novel siRNA delivery technique by constructing a gene circuit

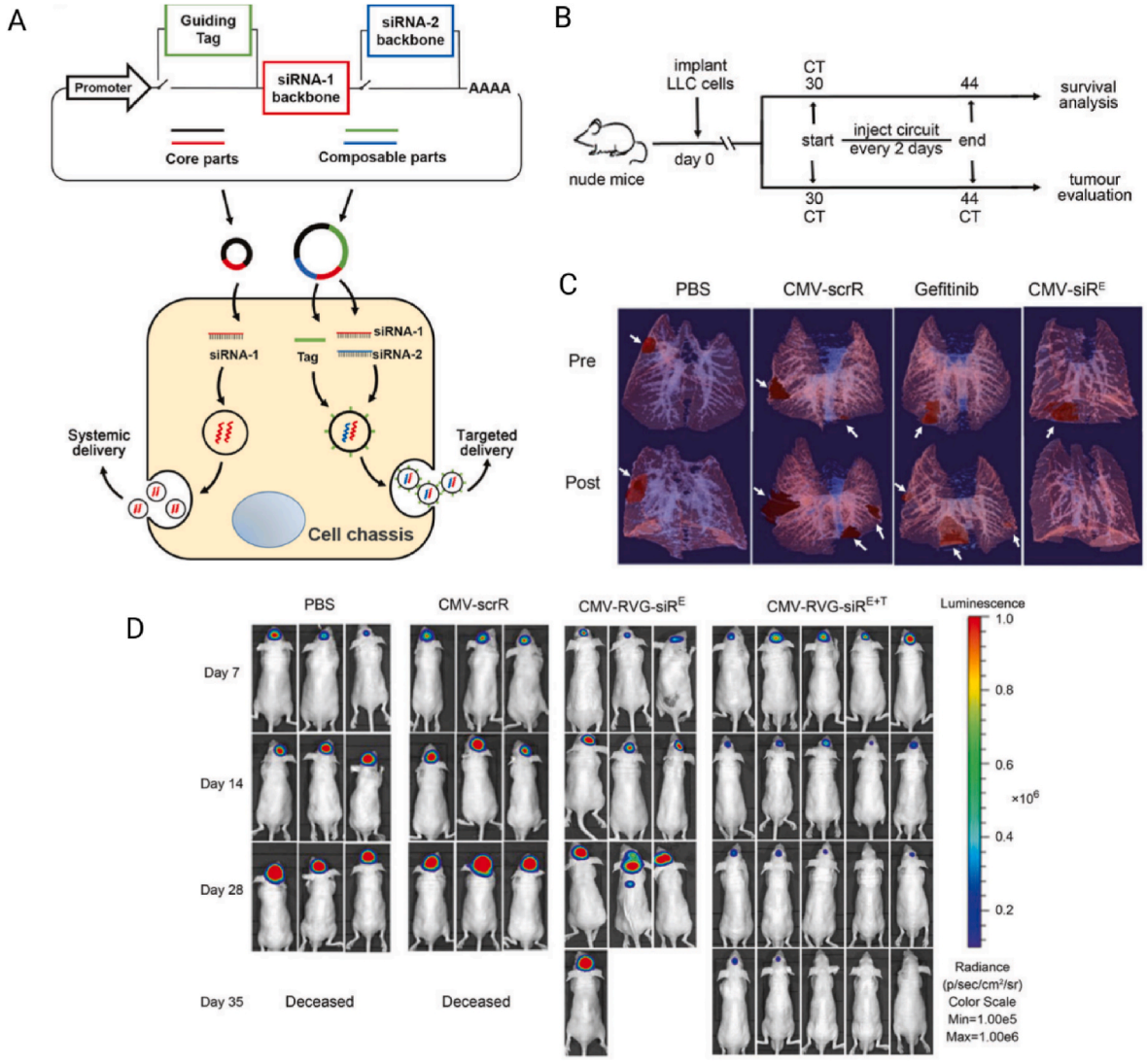

Fig. 6. EVs as Tumor-Targeting Nanocarriers for siRNA Delivery. (A) The genetic circuit consisted of a core promoter driving siRNA expression, with additional modular components for targeting EVs and delivering dual siRNAs to specific tissues. (B) Nude mice were injected with LLC cells, confirmed for lung tumors via micro-CT, and then treated with PBS, CMV-scrR, CMV-siRE circuits, or gefitinib, with survival and tumor growth monitored. (C) 3-D reconstructions of mouse lungs pre- and post-treatment with genetic circuits or gefitinib. (D) Therapeutic effect of mouse glioblastoma. Adapted with permission from Fu et al.[87] Copyright 2021, Springer Nature.

that expresses siRNA *in vitro*. This circuit can guide the synthesis of siRNA in hepatocytes and load it into EVs, enabling flexible insertion of multiple siRNAs and targeting peptides. The team initially used this technique to achieve silencing of the EGFR and KRAS genes in lung cancer. Subsequently, by inserting the RVG targeting peptide along with siRNAs targeting EGFR and TNC into the circuit, they significantly inhibited the growth of glioblastoma[87] (Fig. 6).

Apart from the aforementioned solid tumors, leukemia, as a hematological malignancy with an increasingly younger patient demographic, urgently requires the development of precise and safe therapeutic strategies. It has been demonstrated that the interleukin-3 receptor (IL3-R) is overexpressed in chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) cells. Engineered EVs have been utilized to deliver the Bcr-Abl inhibitor Imatinib (IM) or Bcr-Abl siRNA specifically to CML cells, and effectively overcame pharmacological resistance. EVs containing IL3-Lamp2b and loaded with IM can specifically target tumor cells *in vivo*, thereby inhibiting tumors.[88]

7. EVs as targeted delivery vehicles for other RNA types

In addition to the aforementioned mRNAs, miRNAs, and siRNAs, which have been widely utilized in cancer therapy, there is growing interest in employing other types of RNAs for targeted cancer treatment (Table 1). ASOs are a class of oligonucleotides similar to siRNAs, typically ranging from 16 to 20 nucleotides in length, and primarily function through mechanisms such as mRNA silencing and modulation of RNA splicing. Upon recognizing their target RNAs, ASOs can cleave the target RNAs via RNase H and effectively prevent the following protein expression process. Unlike siRNAs, which generally regulate mRNAs, ASOs can also modulate certain non-coding RNAs within the cell nucleus.[101] Kamerkar et al. engineered EVs to deliver ASOs targeting STAT6 and achieved robust STAT6 gene silencing in the liver. This reprogramming of tumor-associated macrophages (TAMs) into an anti-tumor M1 phenotype significantly inhibited tumor growth in colorectal and liver cancer models.[89] Additionally, anti-microRNA oligonucleotides (AMOs) or anti-miRNAs function by binding to cancer-associated miRNAs to treat tumors.[102] Han et al. developed iRGD-modified mesenchymal stem cell-derived EVs and loaded them with anti-miRNA-221 AMOs via electroporation. The engineered EVs effectively targeted the tumor sites and were internalized by tumor cells through interaction with neuropilin-1 (NRP-1), which significantly suppressed tumor growth.[90] As previously mentioned, EVs modified with RVG peptides for targeting glioblastoma have been widely applied. Kim et al. constructed T7 peptide-modified EVs that bound to transferrin receptors overexpressed on glioblastoma cells. After loading with AMO-21, the results indicated that T7-modified EVs achieved higher delivery efficiency to glioblastoma cells compared to RVG-modified EVs. This approach effectively reduced miR-21 levels in glioblastoma, and induced the expression of (PDCD4) and phosphatase and tensin homolog (PTEN), thereby inhibiting tumor growth.[91] Liang et al. modified the surface of EVs with HER2 and loaded them with the chemotherapeutic drug 5-Fluorouracil (5-FU)[103] and miR-21 inhibitors. Downregulating miR-21 enhanced the expression of PTEN and human DNA MutS homolog 2 (hMSH2) and induced cell cycle arrest. Importantly, this co-delivery strategy reversed drug resistance and significantly enhanced the cytotoxicity of 5-FU, which ultimately led to substantial inhibition of colorectal cancer growth in mice.[92]

In addition, lncRNAs primarily influencing gene expression at the transcriptional and post-transcriptional levels. In a study, the authors explored the use of engineered EVs as targeted delivery vehicles for lncRNA MEG3 in osteosarcoma (OS) therapy. The research showed that MEG3 expression played a significant role in inhibiting OS cell proliferation, migration, and invasion, with its potential to suppress tumor growth. The study also highlighted the development of cRGD-modified exosomes (cRGD-Exo-MEG3), which were engineered to enhance the targeting ability of MEG3 to the αvβ3 integrin on OS cells. The conjugation efficiency of cRGD to Exo-MEG3 was about 35.2 %, and these engineered EVs demonstrated superior tumor-targeting capabilities both *in vitro* and *in vivo*.[104]

As previously discussed, CAR-T therapy has been extensively utilized in targeted cancer treatment. Johnson et al. transfected CAR-T cells with foreign tumor antigens and an RNA agonist RN7SL1 that activated the RIG-I/MDA5 pathway. The antigens and RN7SL1 were subsequently secreted into EVs. The engineered EVs effectively infiltrated into tumors to enhance the presentation of tumor cell antigens while activating myeloid cells and dendritic cells, which hence initiated T cell immune rejection of tumors that had lost CAR antigens.[93] The combination of RN7SL1 RNA with CAR-T cell-derived EVs demonstrated prolonged survival and robust anti-tumor activity, which may provide new insights into RNA-loaded EVs for targeted cancer therapy. This approach highlights the growing potential of RNA-engineered EVs as a versatile platform, capable of delivering functional RNAs to enhance tumor-specific therapeutic efficacy. Building on this concept, subsequent research aims to develop diverse targeting strategies that integrate multiple tumor treatment-related RNAs. By activating key pathways relevant to tumor progression and immune response, these strategies could significantly amplify therapeutic outcomes, marking a pivotal advancement in the field of RNA-based cancer therapeutics.

8. EVs mediated immunotherapy in cancer: clinical trial insights

Recent clinical trials have explored innovative approaches for utilizing engineered EVs to deliver therapeutic agents, such as antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), and tumor antigens, for the treatment of various cancers. These trials demonstrate the potential of EVs based therapies in improving targeting specificity, activating immune responses, and minimizing the side effects commonly associated with conventional cancer treatments. Below are several promising Phase 1 clinical trials that highlight the progress of EVs mediated immunotherapy.

8.1. EVs mediated siRNA delivery for KRAS-Mutant pancreatic cancer

A Phase 1 clinical trial (NCT03608631) evaluated the use of modified EVs to deliver small interfering RNA (siRNA) targeting the KRAS mutation in patients with pancreatic cancer. The KRAS mutation is notoriously difficult to target with conventional therapies. This study demonstrated that 30 % of patients showed a reduction in tumor volume after treatment, with no severe immune reactions observed. The results underscore the potential of EVs mediated siRNA delivery as a targeted, effective approach to treating cancers driven by difficult-to-target genetic mutations such as KRAS mutations, marking a promising step forward in cancer immunotherapy.

8.2. EVs mediated ASO delivery in advanced hepatocellular carcinoma

Another Phase 1 clinical trial (NCT05375604) assessed the delivery of antisense oligonucleotides (ASO-STAT6) using EVs in patients with advanced hepatocellular carcinoma (HCC) and liver metastases from primary gastric and colorectal cancers. The trial demonstrated significant reductions in tumor burden in some patients. The use of EVs to deliver ASOs targeting the STAT6 pathway, which plays a crucial role in tumor immune evasion, represents a novel immunotherapeutic approach. This study supports the concept that EVs mediated RNA delivery can offer therapeutic benefits, particularly in cancers that are resistant to conventional treatments.

8.3. Glioma immunotherapy via EVs mediated tumor antigen delivery

A Phase 1 clinical trial (NCT01550523) investigated a novel immunotherapy strategy for glioma patients. This approach involved harvesting the patient's tumor cells during surgery, treating them with an

antisense molecule (IGF-1R/AS ODN) to induce apoptosis, and re-implanting the treated cells encapsulated in small diffusion chambers in the patient's abdomen. The dying tumor cells released EVs containing tumor antigens, which, in combination with the antisense molecule, activated the immune system to target and destroy the tumor. Early results indicated promising biological responses, including tumor-infiltrating lymphocytes, which suggest the potential for tumor regression. The trial also emphasized the importance of immune activation and the potential of this treatment to provide better outcomes with fewer risks compared to traditional therapies like radiation and chemotherapy.

8.4. *Targeting malignant glioma with engineered EVs*

Another Phase 1 clinical trial (NCT02507583) explored a similar approach for patients with newly diagnosed malignant glioma. In this study, the patient's own tumor cells were harvested, modified with an investigational antisense molecule targeting a surface receptor protein, and then re-implanted encapsulated in diffusion chambers. The trial aimed to activate the immune system through the release of tumor antigens and immune stimulation via the antisense molecule. Initial findings from an earlier trial showed an increase in tumor-infiltrating lymphocytes, suggesting a beneficial immune response. This novel approach seeks to provide a more targeted and less risky alternative to radiation and chemotherapy, offering promising therapeutic outcomes.

These trials collectively highlight the evolving landscape of EVs mediated therapies, demonstrating their potential to offer more targeted, effective, and less toxic treatment options for a range of cancers, including glioma, pancreatic cancer, hepatocellular carcinoma, and cancers with metastatic involvement. The success of these early-stage trials paves the way for larger studies to explore the optimal delivery methods, dosing regimens, and long-term clinical outcomes of EVs based immunotherapies.

9. Future perspectives

To fully realize the potential of EV-mediated RNA delivery, future efforts must focus on interdisciplinary innovations that merge bioengineering, computational modeling, and clinical insights. Hybrid EVs, combining natural tumor-homing properties with synthetic nanoparticles, represent a frontier in precision delivery. For instance, lipid-EV hybrids can integrate pH-responsive release mechanisms, while polymer-EV composites may enhance endosomal escape efficiency. Bioengineered exosomes equipped with multifunctional ligands (e.g., bispecific antibodies, aptamer-peptide chimeras) could enable simultaneous targeting of tumor cells and immune checkpoint molecules, amplifying therapeutic specificity. Scalable biomanufacturing systems, such as 3D dynamic bioreactors mimicking *in vivo* microenvironments or continuous-flow microfluidic platforms, will be critical to meet clinical demands while ensuring batch-to-batch consistency. Furthermore, synergizing EV-based RNA delivery with emerging modalities, such as immune checkpoint inhibitors, photodynamic therapy, and CAR-T cell engineering, could amplify anti-tumor responses and overcome resistance mechanisms. For example, EVs loaded with immunostimulatory RNAs (e.g., RN7SL1) and conjugated with PD-L1 inhibitors may reverse immunosuppressive microenvironments while directly killing tumor cells. By addressing current technical barriers through collaborative innovation, EV-mediated RNA therapies are poised to redefine precision oncology, offering safer, more effective, and clinically viable treatment paradigms.

10. Conclusion

EVs have emerged as a transformative platform for RNA delivery in cancer therapy, leveraging their inherent biocompatibility, low immunogenicity, and capacity for targeted cargo delivery. EV-mediated RNA delivery systems uniquely address critical challenges in conventional therapies, such as RNA instability, off-target effects, and inefficient cellular uptake, by combining natural vesicle properties with engineered enhancements.[105] These systems enable precise tumor targeting through surface modifications and protect therapeutic RNAs from degradation, ensuring effective modulation of oncogenic pathways. The integration of diverse RNA species—including mRNAs, miRNAs, siRNAs, and antisense oligonucleotides—into EVs has demonstrated significant anti-tumor efficacy across multiple cancer models, underscoring their versatility and adaptability in personalized medicine.[106]

Despite these advancements, challenges persist in optimizing targeting specificity within complex tumor microenvironments, improving RNA loading efficiency, and scaling EV production for clinical use.[107] Emerging strategies such as hybrid EV systems, bioengineered exosomes, and advanced bioprocessing technologies (e.g., 3D bioreactors, continuous manufacturing platforms) offer promising solutions to these limitations. Hybrid EVs, which fuse natural EV membranes with synthetic nanoparticles or functionalized lipids, combine the tumor-homing capabilities of natural vesicles with the tunable physicochemical properties of synthetic carriers, enabling enhanced drug loading and programmable targeting. Bioengineered exosomes, generated through genetic modification of parental cells or CRISPR-based editing of EV surface proteins, allow precise control over ligand density and orientation, significantly improving binding affinity to tumor-specific receptors. Scalable bioprocessing systems, including microfluidic-assisted EV isolation, high-throughput electroporation for RNA loading, and automated bioreactor cultures, address production bottlenecks by standardizing EV yield and quality while reducing costs. By refining EV engineering techniques and prioritizing scalable production workflows, the clinical translation of EV-based RNA therapies can be accelerated, ultimately bridging the gap between preclinical innovation and therapeutic application.

CRediT author statement

Ziqi Wang: Writing – original draft, Writing – review & editing. **Mei Lu:** Writing – review & editing. Haonan Xing and Yuanyu Huang involves in discussion and manuscript revision.

Declaration of competing interest

The authors declare no conflicts of interest.

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