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Review Article

Therapeutic Potential of Extracellular Vesicles as Vehicles to Deliver Druggable Molecules for Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and a leading cause of cancer-related deaths. Standard treatments, such as surgery, chemotherapy, and radiotherapy, frequently fail to produce positive therapeutic outcomes. Thus, it is essential to identify new treatment modalities with improved survival rates. Extracellular vesicles (EVs) are nanosized lipid bilayer vesicles secreted by cells that mediate intercellular communication. EVs have been used to deliver several non-coding RNAs (ncRNAs), including miRNA, circRNA, and lncRNA. These ncRNAs demonstrate excellent tumor-suppressive effects and serve as new therapeutic candidates for HCC. EVs possess several characteristics, including high biocompatibility, enhanced stability, and limited cytotoxicity, making them promising drug-delivery vehicles. Although these characteristics make them better drug carriers than traditional synthetic delivery vehicles, translating engineered EVs into clinical practice has been challenging. In this review, we summarise the tumor-suppressive roles of ncRNAs, the recent progress of EV-associated ncRNAs in HCC treatment, unique features of EVs relevant to drug delivery, and current challenges in the clinical translation of EVs.

Introduction

Hepatocellular carcinoma (HCC) is one of the main causes of cancer-related deaths globally and is the most common type of primary liver cancer, accounting for 70–90% of all cases.[1] Hepatic resection and liver transplantation are the most effective curative treatments for HCC. Nonetheless, HCC resection in patients with non-cirrhotic livers has a mortality rate as high as 20%; furthermore, the outcome of liver transplantation is unsatisfactory, with a 10-year survival rate of only 50%. Moreover, hepatic resection is associated with a high recurrence rate of 70% at five years, even in patients with tumor sizes as small as ≤2 cm.[2] Although the recurrence rate following liver transplantation is relatively low, the waiting period for transplantation has been increasing over the years owing to organ shortages, resulting in high patient dropout rates. Finding new treatment modalities with better prognoses is essential for physicians and researchers.

Extracellular vesicles (EVs) are small particles released by almost all cell types in the human body. Numerous names exist across the literature, referring to EVs of various origins, natures, and features.[3] EVs are classified into two major subtypes, microvesicles (MVs) and exosomes. These subtypes have different origins; MVs are formed by budding off the plasma membrane, whereas exosomes are derived from endosomal compartments.[4] Studies have shown that EVs are more than just waste carriers; they are essential in mediating cell-cell communication. EVs carry a variety of components ranging from nucleic acids to proteins, lipids, and metabolites.[5] Notably, cancer cells induce phenotypic

Keywords: Hepatocellular carcinoma; Extracellular vesicles; Exosome; Non-coding RNAs; Drug delivery.

Abbreviations: CAF, cancer-associated fibroblasts; circRNAs, circular RNAs; ECM, extracellular matrix; EVs, extracellular vesicles; HCC, hepatocellular carcinoma; lncRNAs, long ncRNAs; MSCs, mesenchymal stem cells; miRNAs, microRNAs; MVs, microvesicles; ncRNAs, non-coding RNAs; RBPs, RNA-binding proteins.

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reprogramming of recipient cells by transferring molecules contained in EVs. The tumor microenvironment can also be altered by EVs, thereby promoting tumor cell proliferation, invasion, and metastasis.[6,7] The secretion and uptake of EVs involve several steps, from the budding of the donor cell plasma membrane to docking and fusion at the recipient cell surface. Through this process, EV cargoes can enter the recipient cell and subsequently activate signaling pathways that result in various physiological changes.[8] The detailed mechanisms of secretion and uptake of EVs are reviewed elsewhere.[9,10] To date, a substantial amount of literature has reported the secretion of EVs by cancer cells. However, few studies have investigated the uptake of EVs by cancer cells. Pi et al. modified exosomes with folate to enhance their binding to cancer cells with overexpression of folate receptors. The engineered exosomes successfully delivered siRNAs to cancer cells, with a significant suppressive effect on cancer progression.[11] Other studies successfully incorporated miRNA inhibitors (anti-miR-9[12] and anti-miR-214[13]) into EVs to overcome the drug resistance of cancer cells. These findings highlight the feasibility and effectiveness of EVs for drug delivery to cancer cells.

Non-coding RNAs (ncRNAs) are a subtype of RNA not involved in protein-coding. They account for more than 90% of the RNAs made from the human genome and regulate gene expression.[14] Studies have consistently demonstrated that EVs contain several ncRNA species, including microRNAs (miRNAs), circular RNAs (circRNAs), and long ncRNAs (lncRNAs). Furthermore, exosomal ncRNAs participate in cancer regulation.[15–18] Adams et al. showed that *miR-34a* is a tumor-suppressive miRNA that inhibits cancer cell proliferation and invasion and is a direct downstream target of p53.[15] In a large-scale analysis of miRNA profiles from samples, including lung, breast, stomach, prostate, colon, and pancreatic tumors, several miRNAs were significantly downregulated in cancer cells.[16] Lu et al. showed that the tumor-suppressive lncRNA *MEG3* was significantly downregulated in lung cancer cells, subsequently suppressing p53 expression that induces increased cell proliferation.[17] *In vivo* experimental evidence identified *GAS5* as another tumor-suppressive lncRNA that is downregulated in breast cancers.[18] In this review, we discuss the tumor-suppressive functions of ncRNAs and summarise the recent progress in developing EVs as ncRNA delivery vehicles in treating HCC. Additionally, we present the unique features of EVs that make them a good candidate for cancer therapeutics and highlight the current understanding of the challenges associated with the clinical translation of EVs.

Functions of non-coding RNAs in HCC

LncRNAs act as miRNA sponges

Certain lncRNAs interact with miRNAs to act as molecular sponges. They sequester and inhibit the activity of miRNAs, thereby allowing the re-expression of miRNA target genes, which may include important tumor-suppressor genes.[19] Therefore, lncRNAs indirectly regulate cell fate. Zhuang et al. found that *miR-92b* promotes HCC cell proliferation and metastasis by upregulating β-catenin signaling. They further found that Smad7, a direct target of *miR-92b*, was downregulated in HCC cells and that re-expression of Smad7 significantly suppressed *miR-92b*-induced cell proliferation and metastasis. Notably, the lncRNA *XIST* was found to act as a miRNA sponge and inhibit HCC tumourigenesis by targeting *miR-92b.*[20] Wang et al. showed that the lncRNA *SENP3-EIF4A1* acts as a molecular sponge of *miR-9-5p* to protect the expression of the target gene *ZFP36*. Inhibition of *miR-9-5p* subsequently reverses HCC tumourigenesis by inducing apoptosis and reducing the invasiveness and metastatic potential of HCC cells.[21] These studies support the tumor-suppressive role of lncRNAs and their function as miRNA sponges.

lncRNAs in protein regulation and interaction

lncRNAs can associate with proteins to modify their properties and functions. The tumor suppressor p53 is degraded by MDM2 via E3 ubiquitin ligase activity. Zhou et al. found that the p53-MDM2 interaction was decreased in the *lncRNA-PRAL*-overexpressing HCC cells, with a corresponding increase in the HSP90-p53 interaction and p53-induced HCC apoptosis. p53 utilizes HSP90 for its efficient translocation into the nucleus. The interactions of *lncRNA-PRAL* with HSP90 and HSP90 with p53 were confirmed via co-immunoprecipitation, followed by Western blotting. These results revealed that *lncRNA-PRAL* promotes the interaction between HSP90 and p53 and hence, competitively inhibits p53 ubiquitination by MDM2, thereby promoting HCC cell apoptosis.[22] Similarly, Qin et al. found that the lncRNA *PSTAR* could bind to the hnRNP K protein to strengthen its interaction with p53, thereby competitively blocking MDM2-dependent p53 ubiquitination and preventing HCC cells.[23] These studies suggest that lncRNAs act as tumor suppressors by interacting with proteins and modulating their function.

circRNAs act as miRNA sponges

A substantial amount of literature suggests that certain circRNAs have multiple binding sites for miRNAs, indicating that circRNAs can act as miRNA sponges to reverse miRNA-mediated gene degradation.[24] Zhang et al. found that *circTRIM33–12* eliminates the suppression of TET1 by sponging *miR-191*, and the knockdown of TET1 results in HCC cell proliferation, invasion, and migration. The TET1 protein can induce DNA demethylation, thus playing an important role in tumor suppression.[25] Similarly, another group found that *circMTO1* acts as a *miR-9* sponge in HCC cells to liberate downstream p21 expression. Silencing of *circMTO1* in HCC can lead to the downregulation of p21, thereby promoting HCC cell proliferation and invasion.[26] Many other circRNAs (*circ_0091570*, *circ_0014717*, and *circRNA_101505*) were also downregulated in HCC, and they all function as miRNA sponges.[27–29]

circRNAs in protein regulation and interaction

Specific circRNAs can interact with proteins. RNA-binding proteins (RBPs) are necessary for the post-transcriptional modulation of RNAs and the promotion of mRNA stability, localization, and translation. Zhu et al. found that *circZKSCAN1* can act as an RBP (FMRP) sponge rather than a miRNA sponge to inhibit multiple malignant behaviors by suppressing HCC cell stemness. FMRP can bind to the mRNA of its target gene, *CCAR1*, which participates in the Wnt/β-catenin signaling pathway to upregulate cell stemness. *circZKSCAN1* acts as an RBP sponge and blocks the binding between *CCAR1* mRNA and FMRP, thus preventing the transcriptional activity of Wnt/β-catenin signaling.[30] Similarly, Liu et al. found that *circDLC1* can bind to the RBP HuR and prevent its interaction with *MMP1* mRNAs, inhibiting the expression of *MMP1* and preventing HCC progression.[31] On the other hand, Shi et al. identified the role of circRNAs as protein scaffolds. Research has suggested that smaller circRNAs act as scaffolds to facilitate protein binding and interactions that are otherwise physically separated. It was found that *circPABPC1* inhibits HCC cell adhesion and migration by directly binding to and downregulating

oncogenic ITGB1, a key molecule involved in HCC metastasis. *circPABPC1* acts as a bridge to facilitate the interaction between ITGB1 and the proteasome in HCC cells, thus promoting proteasome degradation.[32] Taken together, these studies suggest that circRNAs elicit tumor-suppressive functions by interacting with other proteins.

miRNAs in cell cycle control

Several miRNAs regulate cell cycle progression. Xu et al. found that *miR-195* was significantly downregulated in HCC cells. Overexpression of *miR-195* blocked G1/S transition and suppressed cancer cell proliferation. In terms of molecular mechanisms, *miR-195* directly inhibited cyclin D1 and CDK6, which are required to initiate Rb phosphorylation. Phosphorylated Rb suppresses the inhibition of E2F, which promotes the upregulation of proteins involved in the S-phase entry. Hence, *miR-195* may inhibit HCC progression by repressing Rb-E2F signaling that mediates the G1/S transition.[33] Similarly, Kota et al. found that HCC cells exhibit reduced *miR-26a* expression. *miR-26a* expression induces G1 arrest by directly targeting cyclin D2 and E2, which are essential for the G1/S transition. Overexpression of *miR-26a in vivo* resulted in the inhibition of HCC cell proliferation, induction of apoptosis, and inhibition of cancer progression.[34] The above studies showed that miRNAs play a tumor-suppressive role by inhibiting cell cycle progression in HCC cells.

miRNAs in the regulation of angiogenesis and metastasis

miRNAs are also involved in metastasis and angiogenesis. Tsai et al. found that *miR-122* was significantly downregulated in metastatic HCC. Subsequently, ADAM17 was identified as a direct downstream target of *miR-122*, and its knockdown significantly reduced tumor migration, angiogenesis, and local invasion. This is likely due to the role of ADAM17 in activating certain EGF receptor ligands and modifying integrin signaling during cell adhesion and migration.[35] Fang et al. also found that *miR-29b*-overexpressed HCC cells exhibited a substantial decrease in microvessel density and metastatic potential. *miR-29b* inhibits angiogenesis and metastasis by downregulating MMP2, which assists the migration and proliferation of cancer cells by facilitating the remodeling of the extracellular matrix (ECM) and the release of ECM growth factors.[36] Alpini et al. found that decreased *miR-125b* expression contributed to the invasive phenotype of HCC cells. *miR-125b* suppresses cancer cell survival and tumor angiogenesis by targeting PGF, which may promote the recruitment of circulating hematopoietic progenitor cells and macrophages that contribute to tumor angiogenesis.[37] Taken together, these studies show that miRNAs prevent HCC progression by inhibiting angiogenesis and metastasis (Fig. 1).

EVs loaded with non-coding RNAs as a therapy approach for HCC

EVs can be loaded with therapeutic cargoes such as ncRNAs and delivered to target tumor cells. Several studies have demonstrated that delivering tumor-suppressive ncRNAs via EVs successfully modifies cancer cell activities.[38–43] Various methods have been used to load ncRNAs into EVs. These can be divided into two main categories: endogenous and exogenous. Endogenous loading involves the direct addition of the donor cell ncRNA into EVs before shedding, whereas exogenous loading refers to the loading of the ncRNA into EVs once they are isolated and purified.[38] After loading, the donor and recipient cells are co-cultured to transfer EVs from donor to recipient HCC cells.

MVs derived from mesenchymal stem cells (MSCs) can induce the reprogramming of cancer cells upon the active transfer of ncRNAs. HCC cells treated with MSC-derived MVs showed reduced proliferation and an increased number of cells in the G0-G1 phase. Consistently, increased cell cycle inhibitors were observed in these cells, suggesting that a block in the cell cycle progression led to the observed inhibition of HCC cell proliferation. However, the authors did not further investigate the tumor-suppressive molecules present in these MVs.[39] Alzahrani et al. identified *miR-122* as a liver-specific miRNA downregulated in HCC cells. They reported an increase in the apoptosis of HCC cells following the injection of MSC-derived exosomes loaded with *miR-122*.[40] Similarly, Fonsato et al. showed that the uptake of human liver stem cell-derived MVs by HCC cells resulted in a substantial decrease in tumor cell proliferation and an increase in apoptosis. Several tumor-suppressive miRNAs in MVs have been reported, including *miR451*, *miR223*, *miR24*, *miR125b*, *miR31*, and *miR122*. Notably, proteins essential for cell cycle regulation were downregulated in HCC cells treated with MV, and these proteins are the downstream targets of the antitumor miRNAs present in MV.[41]

Additionally, cancer-associated fibroblasts (CAFs) are essential elements of the tumor microenvironment, which communicate with HCC cells and are, therefore, crucial for HCC therapy. Zhang et al. reported reduced *miR-320a* expression in CAF-derived exosomes. Further investigations suggested that *miR-320a* plays an important tumor-suppressive role by binding to PBX3, suppressing the activation of the MAPK pathway that promotes cell proliferation and metastasis.[42] In another study, Yugawa et al. suggested that CAFs may promote HCC progression via the production of interleukins, chemokines, and other growth factors. Based on this hypothesis, they found that *miR-150-3p* is significantly downregulated in CAF-derived exosomes. Overexpression of *miR-150-3p* in these exosomes significantly inhibited HCC cell migration and invasion. Immunofluorescence further confirmed that *miR-150-3p* was transferred from the CAFs to the HCC cells.[43]

Most studies have identified exosomal miRNAs with antitumor functions; however, other ncRNAs, such as circRNAs and lncRNAs, may also be important. Chen et al. found that patients with HCC exhibit lower *circ-0051443* expression in plasma exosomes than healthy individuals. Transfecting HCC cells with *circ-0051443*-expressing plasmid significantly inhibited the proliferation of cancer cells. The proposed regulatory mechanism is that *circ-0051443* acts as a 'sponge' to sequester *miR-331-3p*, resulting in downstream BAK1 gene transcription, which is important for cell death regulation and mitochondria-mediated apoptosis.[44] Wang et al. identified *lncRNA SENP3*-EIF4A1 as a molecular sponge of *miR-9-5p*, and its expression was significantly reduced in patients with HCC compared to healthy controls. When exosomal *SENP3*-EIF4A1 was transferred from healthy liver cells to HCC cells, apoptosis was increased, and the invasiveness and metastatic potential of HCC cells were decreased.[21] These studies provide evidence for the use of EVs to deliver antitumor ncRNAs in HCC therapy.

Advantages of EV-based drug-delivery system

Safety profiles

Unlike synthetic drug-delivery systems, EVs are relatively safe and minimally reactive to the immune system because of their endogenous origin and high biocompatibility (Table 1). Synthetic

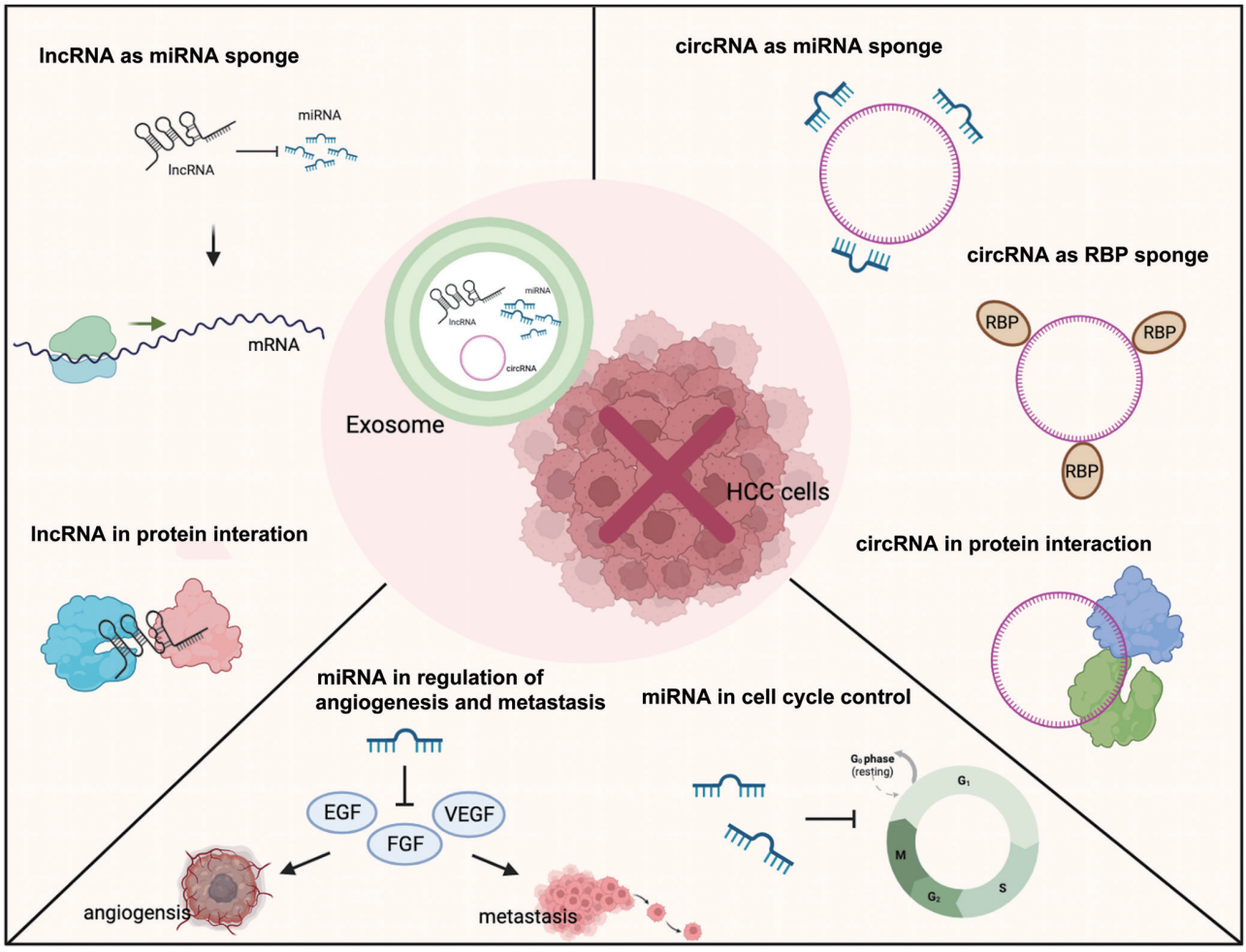

Fig. 1. Exosomes can be used as a drug-delivery vehicle for delivering tumor-suppressive non-coding RNAs (long non-coding RNAs, circular RNAs, and micro RNAs) to HCC cells. HCC, hepatocellular carcinoma; RBP, RNA-binding proteins; circRNA, circular RNAs; lncRNA, long ncRNAs; miRNA, microRNAs.

lipid nanoparticles (such as liposomes) have demonstrated enormous potential for the delivery of nucleic acids, including RNAs. Nevertheless, adverse effects and safety concerns associated with the clinical application of synthetic lipid nanoparticles continue to exist. For instance, poly(ethylene glycol) (PEG), a commonly used hydrophilic polymer coating for drug-delivery vehicles to prevent opsonization and enhance water solubility, is associated with potential side effects. PEG-coated liposomes may induce hypersensitivity reactions, produce toxic side products, and change pharmacokinetic behavior by altering the circulation time of the enclosed

Table 1. Advantages and disadvantages of EV-based different drug-delivery systems

	Advantages	*Disadvantages*
Synthetic lipid nanoparticles	Easy to be modified, controllable size and shape	PEG-coated nanolipids may induce toxic side effects and alter drug pharmacokinetic behavior
	High scalability and reproducibility, easy for quality control	Accumulation of lipids in the liver and spleen may cause pathological changes
	Low manufacturing cost	Not easily targetable
EV-based drug-delivery system	Low immunogenicity	Low scalability and reproducibility, difficult for quality control
	Relatively low cytotoxicity when externally modified	Low drug loading efficiency
	High delivery efficiency due to membrane proteins and lipids that can bind specifically to receptors on target cells	Lack of development in modification techniques

EV, extracellular vesicle; PEG, poly(ethylene glycol).

Table 2. Passive and active loading methods to enhance tumor-targeting ability and drug-delivery efficiency

Strategies	Methods	Description	Advantages	Disadvantages
Passive loading	Simple incubation	EVs are co-incubated with drugs at room temperature	Simple and straightforward	Limited by the volume of EVs and small membrane pore size
Active loading	Electroporation	Uses electrical current to increase the permeability of EV membranes to allow rapid drug entry	High loading efficiency; Ability to load large molecules	Disruption of EV membrane integrity; EV aggregation
	Sonication	Ultrasound probe with different amplitude is used to permeabilize the EV membrane	High loading efficiency	Disruption of EV membrane integrity
	Extrusion	An extruder is used to squeeze the cells co-cultured with the drug to complete drug loading	High loading efficiency; Short duration	Disruption of EV membrane integrity
	Freeze-thaw cycle	Formation of temporary pores on EV membrane through several rapid freeze-thaw cycles	No change in the EV surface charge	Low loading efficiency due to EV aggregation
	Saponin	Form pores on EV membrane through interactions with cholesterol	Higher loading efficiency	Disruption of EV membrane integrity

EV, extracellular vesicle.

drugs.[45] Additionally, it has been noted that synthetic lipid nanoparticles might induce toxic immune responses *in vivo*, causing liver injury in rodents. This might be explained by the cytotoxicity of the lipid materials and their ability to induce a dramatic proinflammatory response.[46] In contrast, Kamerkar et al. showed that exosomes were better siRNA-delivering vehicles than liposomes, as they suppressed KRAS-mutated cancers without inducing any obvious adverse immune responses. Moreover, plasma membrane-like phospholipids and membrane proteins of exosomes may prevent them from being quickly eliminated from circulation.[47] More importantly, no detectable toxicity or inflammatory response was noted, even when exosomes were externally modified with ligands to enhance delivery and uptake.[48] Although some studies have suggested that nanoparticles, including EVs, tend to accumulate in the liver *in vivo*, Saleh et al. confirmed that EVs induced minimal hepatotoxicity and immunogenicity, as the uptake of EVs into HepG2 cells did not show notable changes in histopathology, proinflammatory cytokine levels, or liver transaminases.[49] These findings showed that EVs are generally well-tolerated and may be better candidates than other synthetic drug-delivery systems.

Enhanced drug delivery with modification

EVs can be modified using various strategies to improve their tumor-targeting ability and drug-delivery efficiency. Passive or active loading methods are used to enhance the loading of exogenous cargo. Passive loading refers to the incubation of EVs with therapeutic drugs. This method generally does not damage the structure of EVs and is highly effective for hydrophobic drugs with poor solubility. Active loading refers to loading therapeutic drugs into EVs, mainly by electroporation, sonication, extrusion, freeze-thaw cycles, and saponins. The details of these methods are listed in Table 2. Kim et al. compared different drug loading methods and showed that all active loading methods achieved higher loading efficiencies than passive loading, especially sonication.[50] However, the most appropriate loading method depends on the chemical and physical properties of the cargo. For instance, small and hydrophobic molecules can easily cross the hydrophobic membrane of EVs; hence, coincubation would be suitable in this case. Electroporation and sonication are the best approaches for small RNAs (siRNA and miRNA) that require higher loading efficiencies.[51,52] To facilitate specific binding and uptake by cancer cells, EV surfaces are modified using either covalent or non-covalent methods. Covalent modifications mainly involve chemical conjugation, attaching ligands to the EV surfaces. This method generally does not affect the structural integrity of EVs; however, it depends on carefully controlling the modification conditions (such as temperature, pressure, and pH) to avoid denaturing them.[53] Non-covalent modification involves electrostatic and ligand-receptor interactions. The EV surfaces are negatively charged, thereby allowing the binding of positively charged molecules via electrostatic interactions. Zhan et al. showed that attaching a cationic lipid-sensitive endosomolytic peptide, L17E, to the EVs surface enhanced miRNA release and strongly suppressed tumor progression without apparent adverse effects.[54] In contrast, hydrophobic ligands can be integrated into the EV membranes via hydrophobic interactions. Cheng et al. showed that integrating nuclear localization signal peptides to the EV surfaces greatly enhanced the nuclear delivery of cargo, thus inhibiting tumor growth.[55] Taken together, EVs can be modified using several bioengineering methods to achieve enhanced targetability and drug-delivery efficiency.

Challenges of EV-based drug delivery

Although *in vivo* studies have shown promising progress in EV-based drug delivery, several challenges may hinder the clinical translation and application of EVs. First, a major bottleneck is the massive production of engineered clinical-grade EVs. This refers to the sterile production of EVs in large batches sufficient for clinical testing without batch-to-batch variation and decreased effectiveness. Currently, no viable approach satisfies the desired standards for large-scale EV production. Traditional methods, such as ultracentrifugation, have limitations, including low production yield, non-exosomal contaminants, and poor reproducibility. Large-scale manufacturing of sterile EVs can be achieved using a bioreactor-based culture system and developing a streamline-based microfluidic filtration device for efficient purification.[56,57] Second, achieving a higher drug loading efficiency for EVs is needed. Vader et al. suggested that the loading efficiency of EVs is relatively low compared to that of synthetic liposomes.[58] This may be because EVs develop a high proportion of parental ma-

terials during their formation, leaving a limited loading space for exogenous drugs. Additionally, the loading capacity of EVs may be affected by their various chemical and lipid components; hence, it is important to choose an appropriate method for optimizing the loading efficiency of EVs. The characteristics of each loading method are presented in Table 2. Third, more comprehensive preclinical examinations should be conducted to prevent potential adverse effects, particularly those related to pharmacokinetics, pharmacodynamics, toxicity, and dosage. Some researchers have argued that EVs produced from immortalized cell lines may carry oncogenic materials. However, different immortalized cell lines have been commonly used for EV production owing to their infinite supply and high proliferation rate. As a safe drug carrier, EVs are minimally reactive to the host immune system and are derived from healthy human cells. For example, Zhu et al. showed that administering EVs derived from human embryonic kidney cells to mice for three weeks induced no adverse side effects. The engineered type of EVs showed notable clinical benefits, as the loaded *miR-199a-3p* significantly reduced the proliferation of CD44-positive HCC but not wild types.[59] Moreover, modification and engineering may alter the composition and content of EVs, reducing their effectiveness and immunogenicity. These potential adverse effects should be considered when developing new methods for EV modifications.

Future perspectives

Future studies should explore the therapeutic effects of EVs as ncRNA carriers for targeting HCC cells. To optimize EV use, it is important to understand the underlying mechanisms involved in the cellular sorting of cargoes, which may provide valuable insights into the loading of ncRNAs into EVs. Moreover, EVs from various cell origins may interact differently with the same type of recipient cell; thus, a better understanding of this variation in EV transfer and uptake could improve therapeutic efficacy. Finally, understanding the components of the EV membranes and selecting appropriate modification techniques are essential for EV modifications. This would avoid structural changes to the EV membranes and preserve their physicochemical stability.

Conclusions

EVs have enormous potential as drug-delivery vehicles for cancer therapeutics. In this review, we identified several ncRNAs (miRNAs, circRNAs, and lncRNAs) that play a crucial role in tumor suppression. Several studies have successfully introduced ncRNAs into engineered EVs, significantly inhibiting HCC progression. Targets such as β-catenin, which are not readily targetable by small molecules, can be targeted using siRNA or miRNA; hence, the RNA-targeting approach is an attractive strategy for the modulation of gene expression. Despite all the challenges and questions to be addressed, EVs may still provide hope for developing new treatment modalities against the deadly HCC.

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Conflict of interest

The authors declare no conflict of interest in the publication of this work.

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References

[1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015;65(2):87–108. doi:10.3322/caac.21262, PMID:25651787.

[2] Llovet JM, Kelley RK, Villanueva A, Singal AG, Pikarsky E, Roayaie S, *et al*. Hepatocellular carcinoma. Nat Rev Dis Primers 2021;7(1):6. doi:10.1038/s41572-020-00240-3, PMID:33479224.

[3] Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu Rev Cell Dev Biol 2014;30:255–289. doi:10.1146/annurev-cellbio-101512-122326, PMID:25288114.

[4] Murphy DE, de Jong OG, Brouwer M, Wood MJ, Lavieu G, Schiffelers RM, *et al*. Extracellular vesicle-based therapeutics: natural versus engineered targeting and trafficking. Exp Mol Med 2019;51(3):1–12. doi:10.1038/s12276-019-0223-5, PMID:30872574.

[5] Xavier CPR, Caires HR, Barbosa MAG, Bergantim R, Guimaraes JE, Vasconcelos MH. The Role of Extracellular Vesicles in the Hallmarks of Cancer and Drug Resistance. Cells 2020;9(5):1141. doi:10.3390/cells9051141, PMID:32384712.

[6] Fang T, Lv H, Lv G, Li T, Wang C, Han Q, *et al*. Tumor-derived exosomal miR-1247-3p induces cancer-associated fibroblast activation to foster lung metastasis of liver cancer. Nat Commun 2018;9(1):191.

doi:10.1038/s41467-017-02583-0, PMID:29335551.
[7] Peinado H, Aleckovic M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, *et al*. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med 2012;18(6):883–891. doi:10.1038/nm.2753, PMID:22635005.
[8] Pitt JM, Kroemer G, Zitvogel L. Extracellular vesicles: masters of intercellular communication and potential clinical interventions. J Clin Invest 2016;126(4):1139–1143. doi:10.1172/JCI87316, PMID: 27035805.
[9] van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. Nat Rev Mol Cell Biol 2018;19(4):213–228. doi:10.1038/nrm.2017.125, PMID:29339798.
[10] Mathieu M, Martin-Jaular L, Lavieu G, Thery C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. Nat Cell Biol 2019;21(1):9–17. doi:10.1038/s41556-018-0250-9, PMID:30602770.
[11] Zheng Z, Li Z, Xu C, Guo B, Guo P. Folate-displaying exosome mediated cytosolic delivery of siRNA avoiding endosome trapping. J Control Release 2019;311-312:43–49. doi:10.1016/j.jconrel.2019.08.021, PMID:31446085.
[12] Munoz JL, Bliss SA, Greco SJ, Ramkissoon SH, Ligon KL, Rameshwar P. Delivery of Functional Anti-miR-9 by Mesenchymal Stem Cell-derived Exosomes to Glioblastoma Multiforme Cells Conferred Chemosensitivity. Mol Ther Nucleic Acids 2013;2:e126. doi:10.1038/mtna.2013.60, PMID:24084846.
[13] Wang X, Zhang H, Bai M, Ning T, Ge S, Deng T, *et al*. Exosomes Serve as Nanoparticles to Deliver Anti-miR-214 to Reverse Chemoresistance to Cisplatin in Gastric Cancer. Mol Ther 2018;26(3):774–783. doi:10.1016/j.ymthe.2018.01.001, PMID:29456019.
[14] Slack FJ, Chinnaiyan AM. The Role of Non-coding RNAs in Oncology. Cell 2019;179(5):1033–1055. doi:10.1016/j.cell.2019.10.017, PMID:31730848.
[15] Adams BD, Parsons C, Slack FJ. The tumor-suppressive and potential therapeutic functions of miR-34a in epithelial carcinomas. Expert Opin Ther Targets 2016;20(6):737–753. doi:10.1517/14728222.2016.1114102, PMID:26652031.
[16] Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, *et al*. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 2006;103(7):2257–2261. doi:10.1073/pnas.0510565103, PMID:16461460.
[17] Lu KH, Li W, Liu XH, Sun M, Zhang ML, Wu WQ, *et al*. Long non-coding RNA MEG3 inhibits NSCLC cells proliferation and induces apoptosis by affecting p53 expression. BMC Cancer 2013;13:461. doi:10.1186/1471-2407-13-461, PMID:24098911.
[18] Zhang Z, Zhu Z, Watabe K, Zhang X, Bai C, Xu M, *et al*. Negative regulation of lncRNA GAS5 by miR-21. Cell Death Differ 2013;20(11):1558–1568. doi:10.1038/cdd.2013.110, PMID:23933812.
[19] Huang Z, Zhou JK, Peng Y, He W, Huang C. The role of long noncoding RNAs in hepatocellular carcinoma. Mol Cancer 2020;19(1):77. doi:10.1186/s12943-020-01188-4, PMID:32295598.
[20] Zhuang LK, Yang YT, Ma X, Han B, Wang ZS, Zhao QY, *et al*. MicroRNA-92b promotes hepatocellular carcinoma progression by targeting Smad7 and is mediated by long non-coding RNA XIST. Cell Death Dis 2016;7:e2203. doi:10.1038/cddis.2016.100, PMID:27100897.
[21] Wang J, Pu J, Zhang Y, Yao T, Luo Z, Li W, *et al*. Exosome-transmitted long non-coding RNA SENP3-EIF4A1 suppresses the progression of hepatocellular carcinoma. Aging (Albany NY) 2020;12(12):11550–11567. doi:10.18632/aging.103302, PMID:32602848.
[22] Zhou CC, Yang F, Yuan SX, Ma JZ, Liu F, Yuan JH, *et al*. Systemic genome screening identifies the outcome associated focal loss of long noncoding RNA PRAL in hepatocellular carcinoma. Hepatology 2016;63(3):850–863. doi:10.1002/hep.28393, PMID:26663434.
[23] Qin G, Tu X, Li H, Cao P, Chen X, Song J, *et al*. Long Noncoding RNA p53-Stabilizing and Activating RNA Promotes p53 Signaling by Inhibiting Heterogeneous Nuclear Ribonucleoprotein K deSUMOylation and Suppresses Hepatocellular Carcinoma. Hepatology 2020;71(1):112–129. doi:10.1002/hep.30793, PMID:31148184.
[24] Zhang Y, Wang Y. Circular RNAs in Hepatocellular Carcinoma: Emerging Functions to Clinical Significances. Front Oncol 2021;11:667428. doi:10.3389/fonc.2021.667428, PMID:34055634.
[25] Zhang PF, Wei CY, Huang XY, Peng R, Yang X, Lu JC, *et al*. Circular RNA circTRIM33-12 acts as the sponge of MicroRNA-191 to suppress hepatocellular carcinoma progression. Mol Cancer 2019;18(1):105. doi:10.1186/s12943-019-1031-1, PMID:31153371.
[26] Han D, Li J, Wang H, Su X, Hou J, Gu Y, *et al*. Circular RNA circMTO1 acts as the sponge of microRNA-9 to suppress hepatocellular carcinoma progression. Hepatology 2017;66(4):1151–1164. doi:10.1002/hep.29270, PMID:28520103.
[27] Wang YG, Wang T, Ding M, Xiang SH, Shi M, Zhai B. hsa_circ_0091570 acts as a ceRNA to suppress hepatocellular cancer progression by sponging hsa-miR-1307. Cancer Lett 2019;460:128–138. doi:10.1016/j.canlet.2019.06.007, PMID:31207319.
[28] Ma H, Huang C, Huang Q, Li G, Li J, Huang B, *et al*. Circular RNA circ_0014717 Suppresses Hepatocellular Carcinoma Tumorigenesis Through Regulating miR-668-3p/BTG2 Axis. Front Oncol 2020;10:592884. doi:10.3389/fonc.2020.592884, PMID:33598424.
[29] Luo Y, Fu Y, Huang R, Gao M, Liu F, Gui R, *et al*. CircRNA_101505 sensitizes hepatocellular carcinoma cells to cisplatin by sponging miR-103 and promotes oxidored-nitro domain-containing protein 1 expression. Cell Death Discov 2019;5:121. doi:10.1038/s41420-019-0202-6, PMID:31372241.
[30] Zhu YJ, Zheng B, Luo GJ, Ma XK, Lu XY, Lin XM, *et al*. Circular RNAs negatively regulate cancer stem cells by physically binding FMRP against CCAR1 complex in hepatocellular carcinoma. Theranostics 2019;9(12):3526–3540. doi:10.7150/thno.32796, PMID:31281495.
[31] Liu H, Lan T, Li H, Xu L, Chen X, Liao H, *et al*. Circular RNA circDLC1 inhibits MMP1-mediated liver cancer progression via interaction with HuR. Theranostics 2021;11(3):1396–1411. doi:10.7150/thno.53227, PMID:33391541.
[32] Shi L, Liu B, Shen DD, Yan P, Zhang Y, Tian Y, *et al*. A tumor-suppressive circular RNA mediates uncanonical integrin degradation by the proteasome in liver cancer. Sci Adv 2021;7(13):abe5043. doi:10.1126/sciadv.abe5043, PMID:33762338.
[33] Xu T, Zhu Y, Xiong Y, Ge YY, Yun JP, Zhuang SM. MicroRNA-195 suppresses tumorigenicity and regulates G1/S transition of human hepatocellular carcinoma cells. Hepatology 2009;50(1):113–121. doi:10.1002/hep.22919, PMID:19441017.
[34] Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, *et al*. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell 2009;137(6):1005–1017. doi:10.1016/j.cell.2009.04.021, PMID:19524505.
[35] Tsai WC, Hsu PW, Lai TC, Chau GY, Lin CW, Chen CM, *et al*. MicroRNA-122, a tumor suppressor microRNA that regulates intrahepatic metastasis of hepatocellular carcinoma. Hepatology 2009;49(5):1571–1582. doi:10.1002/hep.22806, PMID:19296470.
[36] Fang JH, Zhou HC, Zeng C, Yang J, Liu Y, Huang X, *et al*. MicroRNA-29b suppresses tumor angiogenesis, invasion, and metastasis by regulating matrix metalloproteinase 2 expression. Hepatology 2011;54(5):1729–1740. doi:10.1002/hep.24577, PMID:21793034.
[37] Alpini G, Glaser SS, Zhang JP, Francis H, Han Y, Gong J, *et al*. Regulation of placenta growth factor by microRNA-125b in hepatocellular cancer. J Hepatol 2011;55(6):1339–1345. doi:10.1016/j.jhep.2011.04.015, PMID:21703189.
[38] Sutaria DS, Badawi M, Phelps MA, Schmittgen TD. Achieving the Promise of Therapeutic Extracellular Vesicles: The Devil is in Details of Therapeutic Loading. Pharm Res 2017;34(5):1053–1066. doi:10.1007/s11095-017-2123-5, PMID:28315083.
[39] Bruno S, Collino F, Deregibus MC, Grange C, Tetta C, Camussi G. Microvesicles derived from human bone marrow mesenchymal stem cells inhibit tumor growth. Stem Cells Dev 2013;22(5):758–771. doi:10.1089/scd.2012.0304, PMID:23034046.
[40] Alzahrani FA, El-Magd MA, Abdelfattah-Hassan A, Saleh AA, Saadeldin IM, El-Shetry ES, *et al*. Potential Effect of Exosomes Derived from Cancer Stem Cells and MSCs on Progression of DEN-Induced HCC in Rats. Stem Cells Int 2018;2018:8058979. doi:10.1155/2018/8058979, PMID:30224923.
[41] Fonsato V, Collino F, Herrera MB, Cavallari C, Deregibus MC, Cisterna B, *et al*. Human liver stem cell-derived microvesicles inhibit hepatoma growth in SCID mice by delivering antitumor microRNAs. Stem Cells 2012;30(9):1985–1998. doi:10.1002/stem.1161, PMID:22736596.
[42] Zhang Z, Li X, Sun W, Yue S, Yang J, Li J, *et al*. Loss of exosomal miR-320a from cancer-associated fibroblasts contributes to HCC prolif-

eration and metastasis. Cancer Lett 2017;397:33–42. doi:10.1016/j.canlet.2017.03.004, PMID:28288874.
[43] Yugawa K, Yoshizumi T, Mano Y, Itoh S, Harada N, Ikegami T, *et al*. Cancer-associated fibroblasts promote hepatocellular carcinoma progression through downregulation of exosomal miR-150-3p. Eur J Surg Oncol 2021;47(2):384–393. doi:10.1016/j.ejso.2020.08.002, PMID:32883551.
[44] Chen W, Quan Y, Fan S, Wang H, Liang J, Huang L, *et al*. Exosome-transmitted circular RNA hsa_circ_0051443 suppresses hepatocellular carcinoma progression. Cancer Lett 2020;475:119–128. doi:10.1016/j.canlet.2020.01.022, PMID:32014458.
[45] Knop K, Hoogenboom R, Fischer D, Schubert US. Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives. Angew Chem Int Ed Engl 2010;49(36):6288–6308. doi:10.1002/anie.200902672, PMID:20648499.
[46] Kedmi R, Ben-Arie N, Peer D. The systemic toxicity of positively charged lipid nanoparticles and the role of Toll-like receptor 4 in immune activation. Biomaterials 2010;31(26):6867–6875. doi:10.1016/j.biomaterials.2010.05.027, PMID:20541799.
[47] Kamerkar S, LeBleu VS, Sugimoto H, Yang S, Ruivo CF, Melo SA, *et al*. Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. Nature 2017;546(7659):498–503. doi:10.1038/nature22341, PMID:28607485.
[48] Gao X, Ran N, Dong X, Zuo B, Yang R, Zhou Q, *et al*. Anchor peptide captures, targets, and loads exosomes of diverse origins for diagnostics and therapy. Sci Transl Med 2018;10(444):aat0195. doi:10.1126/scitranslmed.aat0195, PMID:29875202.
[49] Saleh AF, Lazaro-Ibanez E, Forsgard MA, Shatnyeva O, Osteikoetxea X, Karlsson F, *et al*. Extracellular vesicles induce minimal hepatotoxicity and immunogenicity. Nanoscale 2019;11(14):6990–7001. doi:10.1039/c8nr08720b, PMID:30916672.
[50] Kim MS, Haney MJ, Zhao Y, Mahajan V, Deygen I, Klyachko NL, *et al*. Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells. Nanomedicine 2016;12(3):655–664. doi:10.1016/j.nano.2015.10.012, PMID:26586551.
[51] Lamichhane TN, Jeyaram A, Patel DB, Parajuli B, Livingston NK, Arumugasaamy N, *et al*. Oncogene Knockdown via Active Loading of Small RNAs into Extracellular Vesicles by Sonication. Cell Mol Bioeng 2016;9(3):315–324. doi:10.1007/s12195-016-0457-4, PMID:27800035.
[52] Lamichhane TN, Raiker RS, Jay SM. Exogenous DNA Loading into Extracellular Vesicles via Electroporation is Size-Dependent and Enables Limited Gene Delivery. Mol Pharm 2015;12(10):3650–3657. doi:10.1021/acs.molpharmaceut.5b00364, PMID:26376343.
[53] Zhang F, Guo J, Zhang Z, Duan M, Wang G, Qian Y, *et al*. Application of engineered extracellular vesicles for targeted tumor therapy. J Biomed Sci 2022;29(1):14. doi:10.1186/s12929-022-00798-y, PMID:35189894.
[54] Zhan Q, Yi K, Qi H, Li S, Li X, Wang Q, *et al*. Engineering blood exosomes for tumor-targeting efficient gene/chemo combination therapy. Theranostics 2020;10(17):7889–7905. doi:10.7150/thno.45028, PMID:32685027.
[55] Cheng H, Fan JH, Zhao LP, Fan GL, Zheng RR, Qiu XZ, *et al*. Chimeric peptide engineered exosomes for dual-stage light guided plasma membrane and nucleus targeted photodynamic therapy. Biomaterials 2019;211:14–24. doi:10.1016/j.biomaterials.2019.05.004, PMID:31078049.
[56] Mendt M, Kamerkar S, Sugimoto H, McAndrews KM, Wu CC, Gagea M, *et al*. Generation and testing of clinical-grade exosomes for pancreatic cancer. JCI Insight 2018;3(8):e99263. doi:10.1172/jci.insight.99263, PMID:29669940.
[57] Zhang X, Zhang H, Gu J, Zhang J, Shi H, Qian H, *et al*. Engineered Extracellular Vesicles for Cancer Therapy. Adv Mater 2021;33(14):e2005709. doi:10.1002/adma.202005709, PMID:33644908.
[58] Vader P, Mol EA, Pasterkamp G, Schiffelers RM. Extracellular vesicles for drug delivery. Adv Drug Deliv Rev 2016;106(Pt A):148–156. doi:10.1016/j.addr.2016.02.006, PMID:26928656.
[59] Zhu X, Badawi M, Pomeroy S, Sutaria DS, Xie Z, Baek A, *et al*. Comprehensive toxicity and immunogenicity studies reveal minimal effects in mice following sustained dosing of extracellular vesicles derived from HEK293T cells. J Extracell Vesicles 2017;6(1):1324730. doi:10.1080/20013078.2017.1324730, PMID:28717420.