EXTRACELLULAR VESICLES

Advances in therapeutic applications of extracellular vesicles

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Oscar P. B. Wiklander[1,2]*, Meadhbh Á. Brennan[3,4,5], Jan Lötvall[6], Xandra O. Breakefield[4], Samir EL Andaloussi[1,2]*

Extracellular vesicles (EVs) are nanometer-sized, lipid membrane–enclosed vesicles secreted by most, if not all, cells and contain lipids, proteins, and various nucleic acid species of the source cell. EVs act as important mediators of intercellular communication that influence both physiological and pathological conditions. Given their ability to transfer bioactive components and surmount biological barriers, EVs are increasingly being explored as potential therapeutic agents. EVs can potentiate tissue regeneration, participate in immune modulation, and function as potential alternatives to stem cell therapy, and bioengineered EVs can act as delivery vehicles for therapeutic agents. Here, we cover recent approaches and advances of EV-based therapies.

INTRODUCTION

Extracellular vesicles (EVs) comprise a highly conserved and advanced system of intercellular communication, by which cells can exchange information in the form of lipids, proteins, or nucleic acid species. EVs were originally found to be involved in bone mineralization, as well as platelet function, and were called "platelet dust" (*1*). In the early 1980s, two separate publications described that exosomes, a subtype of EVs, can also help discard molecules that a cell does not need (*2*, *3*). In these studies, reticulocytes expelled transferrin receptor in exosomes during their maturation to erythrocytes. Subsequent studies in the 1990s showed that EVs were highly regulatory in the immune system (*4*), and another decade later, it became evident that they were also able to shuttle proteins and RNA between cells (*5*–*7*). Over the past 5 years, research has started to shed light on the various mechanisms by which EVs can regulate biological functions, which span from tissue homeostasis and regulation of inflammation to the growth and metastasis of tumors. In view of their exceptionally broad biological functions and their ability to shuttle large molecules between cells, EVs offer a unique platform for the development of a new class of therapeutics.

EVs are present in all body fluids and are released by all types of cells in the human body. Classically, EVs have been divided into exosomes, smaller vesicles that are released from the interior of any cell via the multivesicular endosomal pathway, and microvesicles that are released from cells by budding of its surface membrane (*8*, *9*). A third, less studied subgroup of EVs, known as apoptotic bodies, are formed by blebbing of dying cells and may contain diverse parts of the cell (*10*). In this Review, we focus on the first two classes of EVs. Until now, scientists based these classifications on EVs prepared by differential centrifugation, with "microvesicles" typically being isolated by a 10,000*g* to 20,000*g* centrifugation and the "exosomes" by a very high speed centrifugation at or above 100,000*g* (*11*). Preparations of microvesicles and exosomes are different in many ways, although there are overlaps in size and content (*12*). They contain distinct proteins and RNA cargo, which suggests that they mediate various biological functions through different molecular mechanisms. Current research indicates that further subdivisions of EVs may be needed to differentiate subtypes, for example, mitochondrial protein-enriched EVs (*13*) and different types of exosomes (*12*).

When developing an EV therapeutic, the first consideration is the cellular source. Thus, EVs from inflammatory cells naturally mediate different biological functions than EVs from mesenchymal stromal cells (MSCs). Multiple efforts are now ongoing in developing MSC-EVs as therapeutics, and multiple experimental studies report that EVs from MSCs mimic the immunoregulatory function and the regenerative capacity of MSCs (Table 1). Culture conditions, yield, and manufacturability are important aspects to consider that will be discussed in this Review but also are extensively discussed in another recent review (*14*). To overcome issues related to mammalian cell EVs, several research groups have also started to manufacture EVs from different types of fruit or vegetables, including ginger, grapes, and lemons (*15*–*17*), and it has been shown that these can be loaded with small molecular cargos, such as methotrexate, and mediate therapeutic effects in animal models (*18*).

Therapeutic EVs may also be modified by using molecular engineering techniques. Such engineered EVs may mediate biological functions in fundamentally different ways. EVs can be loaded exogenously by incorporating cargo on or in isolated EVs or endogenously, in which the cargo is introduced into or generated by the producer cell to exploit the cellular machinery for cargo sorting into EVs (Fig. 1). EVs could be loaded with therapeutic RNA molecules (*19*, *20*) or proteins (*21*) to be delivered to the inside of recipient cells. Alternatively, therapeutic EVs could be engineered to express specific surface molecules, such as biologically active proteins that mediate a specific biological function or a molecule that can neutralize circulating bioactive molecules. Surface ligands can also be used to target EVs to specific recipient cell types, which can facilitate crossing of physiologic barriers, such as the blood-brain barrier (BBB), when targeting neurons (*19*). Other additions to the EV surface could enable fusion with the plasma membrane of the recipient cell or facilitate cytoplasmic release of cargo after endosomal uptake. Last, the route of administration of EVs influences their biodistribution (*22*), which needs to be considered when developing any therapeutic modality to be used in patients.

[1]Department of Laboratory Medicine, Karolinska Institutet, 141 86 Stockholm, Sweden. [2]Evox Therapeutics Limited, Medawar Centre, Robert Robinson Avenue, Oxford OX4 4HG, UK. [3]Harvard School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138, USA. [4]Departments of Neurology and Radiology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02129, USA. [5]INSERM UMR 1238, PhyOS, Faculty of Medicine, Université de Nantes, 44034 Nantes cedex 1, France. [6]Krefting Research Centre, Institute of Medicine at Sahlgrenska Academy, University of Gothenburg, 405 30 Gothenburg, Sweden.
*Corresponding author. Email: samir.el-andaloussi@ki.se (S.EL.A.); oscar.wiklander@ki.se (O.P.B.W.)

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Table 1. Recent disease treatment and tissue regeneration with EVs derived from MSCs. BM, bone marrow; ESC-MSCs, embryonic stem cell–derived MSCs; hiPSCs, human induced pluripotent stem cells; IL-10, interleukin-10; NK, natural killer; PEG, polyethylene glycol; SEC, size exclusion chromatography; TFF, tangential flow filtration; TNF-α, tumor necrosis factor–α; VEGF, vascular endothelial growth factor; UC, ultracentrifugation.

Indication	EV source	Isolation method	Outcome in target disease/ injured tissue
Respiratory			
Pulmonary hypertension	Human umbilical cord Wharton's jelly MSCs	Ultrafiltration followed by PEG precipitation and SEC or by UC	In a murine model of hypoxia-induced pulmonary hypertension, MSC-derived EVs inhibited pulmonary infiltration of macrophages and suppressed production of proinflammatory and pro-proliferative factors. CM depleted of EVs had no effect (*29*).
Neonatal hyperoxic lung injury	Human umbilical cord blood MSCs	Differential centrifugation with UC	MSC-derived EVs were as effective as parental MSCs in attenuating both H_2O_2-induced cell death in rat lung epithelial L2 cells in vitro and hyperoxic lung injuries in vivo. VEGF mRNA and protein within MSC-derived EVs were identified as the critical paracrine factors responsible (*30*).
Acute respiratory illness	Swine BM MSCs	Differential centrifugation with UC	In a pig model of influenza virus, intratracheal administration of MSC-EVs reduced virus shedding, influenza virus replication in the lungs, virus-induced production of proinflammatory cytokines, and influenza virus–induced lung lesions (*31*).
Renal			
Acute kidney injury (AKI)	Human BM MSCs	Differential centrifugation with UC	In a mouse model of glycerol-induced AKI, the administration of MSC-EVs accelerated functional recovery by inducing the proliferation of tubular cells. Ribonuclease treatment abolished the therapeutic benefit, suggesting that this effect was mediated by horizontal transfer of mRNA (*32*).
Kidney inflammation	Swine adipose MSCs	Differential centrifugation with UC	In a porcine model of metabolic syndrome and renal artery stenosis, MSC-EVs attenuated renal inflammation and improved medullary oxygenation and fibrosis. The reno-protective effects of MSC-EVs were attributed to vesicular IL-10 (*33*).
Renal ischemic reperfusion injury	Human umbilical cord MSCs	Differential centrifugation with UC	MSC-EVs improved tubular injury and protected renal functions after acute kidney injury in rats by a process involving the modulation of NK cells (*34*).
Hepatic			
Hepatic injury	Human and murine BM MSCs	Differential centrifugation with UC	In a lethal murine model of hepatic failure induced by D-galactosamine/ TNF-α, MSC-EVs reduced hepatic injury, modulated cytokine expression, and increased survival (*35*).
Liver fibrosis	Human umbilical cord MSCs	Differential centrifugation with UC on a sucrose cushion	MSC-EVs ameliorated carbon tetrachloride (CCl_4)–induced liver fibrosis in mice by inhibiting epithelial-to-mesenchymal transition and protecting hepatocytes (*36*).
Neurological			
Global cerebral ischemia	Murine adipose and BM MSCs	ExoQuick TC kit (Systems Biosciences)	MSC-EVs restored basal synaptic transmission and synaptic plasticity and improved spatial learning and memory in mice (*37*).
Traumatic brain injury (TBI)	Human BM MSCs	Chromatography	MSC-EVs administered after induction of TBI in mice rescued pattern separation and spatial learning impairments (*38*).
Acute spinal cord injury (SCI)	Human BM MSCs	TFF	MSC-EVs attenuated neuroinflammation and improved functional recovery in a rat model of SCI (*39*).
Musculoskeletal			
Osteoarthritis (OA)	Murine BM MSCs	Differential centrifugation with UC	In a collagenase-induced OA model, MSC-EVs protected mice from joint damage (prevented both cartilage and bone degradation) (*40*).
Inflammatory arthritis	Mouse BM MSCs	Differential centrifugation with UC	MSC-EVs exerted an anti-inflammatory role on T and B lymphocytes in vitro and suppressed inflammation in vivo, with smaller-sized EVs exerting a more efficient response (*41*).
Osteochondral defects	Human ESC-MSCs	TFF, sucrose density gradient UC	MSC-EVs completely regenerated osteochondral defects in a rat model after 12 weeks (*42*).
Bone fractures	Human BM MSCs	Differential centrifugation with UC	MSC-EVs enhanced fracture healing in a mouse femoral bone fracture model. A similar therapeutic effect was observed with CM; however, the bone healing effect was abolished by depleting the CM of EVs (*43*).
Osteoporotic bone fractures	hiPSC-MSCs	Ultrafiltration and UC	MSC-EVs enhanced bone regeneration and angiogenesis in critical-sized calvarial defects in ovariectomized rats in a dose-dependent manner (*44*).

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Indication	EV source	Isolation method	Outcome in target disease/injured tissue
Cardiovascular			
Myocardial infarction (MI)	Rat BM MSCs	Total exosome isolation reagent (Invitrogen)	MSC-EVs reduced apoptosis and the myocardial infarct size and up-regulated myocardial LC3B expression as well as improved heart function in rat models of myocardial ischemia reperfusion injury (*45*).
MI	Human ESC-MSCs	TFF followed by sucrose density gradient UC	MSC-EVs reduced myocardial ischemia/reperfusion injury in a mouse model of MI (*28*).
Critical limb ischemia	Mouse BM MSCs	Differential centrifugation with iodixanol gradients UC	Administration of MSC-EVs to mice in vivo increased both blood reperfusion and the formation of new blood vessels and accelerated recovery of hindlimb ischemia (*46*).

Over the last few decades, biological medications, such as monoclonal antibodies and cell therapies including chimeric antigen receptor (CAR) T cells, have achieved tremendous advances in managing disease. We will discuss here why we think EVs are likely to be the next breakthrough in medical treatment and why well-designed EV therapeutics may help to manage and cure disease.

INNATE THERAPEUTIC POTENTIAL OF EVs

Exogenously supplied MSCs derived from different tissues including bone marrow, adipose tissue, and umbilical cord confer therapeutic benefit in a variety of diseases and have achieved success particularly in tissue regeneration (*23*). The initial hypothesis that MSCs, through cellular differentiation, would replace damaged tissue was partially abandoned following observations that very few, if any, cells stably engraft in the host (*24*, *25*). The therapeutic benefits were instead suggested to be imparted by the secretome of MSCs, a hypothesis that was strengthened by observations that MSC-CM (conditioned media) could achieve therapeutic efficacy similar to that realized by MSC administration in many paradigms (*26*, *27*). This has led to the concept of using the MSC secretome (a mixture consisting of EVs and paracrine soluble factors that may be separated from or associated with the EVs) as an alternative to direct MSC therapy in regenerative medicine. The CM contains the MSC secretome, and the therapeutic efficacy of MSC-CM can be mainly attributed to the constituent EVs within (*28*). EVs derived from MSCs have been reported to have therapeutic potential in preclinical studies in diverse tissues and indications, including the treatment of diseases and regenerative medicine targeting the lungs (*29*–*31*), kidney (*32*–*34*), liver (*35*, *36*), central nervous system (*37*–*39*), cartilage (*40*–*42*), bone (*43*, *44*), and heart (*28*, *45*) (Table 1). However, the therapeutic potential of MSC-EVs is still controversial because of the complexity of MSCs regarding tissue origin and cell culture conditions. In addition, the isolation and purity of EVs in relation to other factors in the MSC secretome, including the potential co-isolation of contaminating proteins and nucleic acids, may result in invalid conclusions of EV content and function. The underlying mechanisms attributed to the therapeutic action of MSC-EVs by the transfer of their cargo, as well as the triggering of signaling pathways via cell surface interactions, are diverse and include mitigating or eliciting immune responses, reducing inflammation, inhibiting apoptosis, minimizing oxidative stress, stimulating wound repair, and promoting angiogenesis, which together act to ultimately ameliorate the adverse effects of diseases, promote healing, and restore function (*28*–*46*).

The field has mainly focused on MSC-derived EVs. However, similar to MSCs, a number of different cell types with stem cell–like properties are associated with potential immunomodulatory effects that could be harnessed for therapeutic applications. EVs derived from other regenerative and immunomodulatory cell sources, such as amniotic epithelial cells, endothelial progenitor cells, embryonic stem cells, induced pluripotent stem cells, cardiosphere-derived cells, and dendritic cells (DCs), have been reported to mediate therapeutic effects in preclinical models of wound healing (*47*, *48*), pulmonary fibrosis (*49*), vascular repair (*50*), myocardial infarction (*51*–*53*), and vaccination (*54*). The regenerative capacities and immunomodulatory effects of stem cells have been shown to be dependent on various factors, including donor-associated effects and tissue of origin (*55*, *56*), and these effects may likely extend to their secreted EVs. In addition, culture conditions affect the composition and function of cells and their EVs. Cells exposed to stress-induced conditions, such as oxidative stress (*57*), acidic conditions (*58*), serum starvation (*59*), hypoxia (*60*), ultraviolet (UV) light (*57*), irradiation (*61*), or cell-stimulating substances (*62*), generate varying numbers of EVs with a different composition and function as compared to EVs isolated from cells under normal culture conditions. It is, however, questionable how representative the common cell flask culturing conditions are to physiologically relevant conditions. Three-dimensional (3D) cell culturing in bioreactors, on spheres, or in organoids, believed to mimic the physiological cell conditions better than 2D cultures, gives rise to EVs with altered properties compared to corresponding EVs derived from cells grown as monolayers on flat plastic dishes (*63*, *64*).

Direct comparisons of the efficacy of parental cell therapy with EV administration are lacking, partly due to MSCs' potential to provide a long-term source of EVs on site and the fact that EV injection may or may not be on site and/or recapitulate the number and length of action of EVs released by resident MSCs. MSC-EVs appear to be as effective as their parental MSCs in attenuating hyperoxic lung injuries or mitigating lung inflammation (*30*); however, others have shown minimal potency of MSC-EVs compared to MSC therapy for bone regeneration (*65*). Nevertheless, on the weight of the considerable evidence of their therapeutic utility amassed in preclinical studies, EVs are now being explored by various commercial entities for clinical translation. There are additional logistical advantages of using EVs, which can be considered as an off-the-shelf product, and EVs are also likely to have reduced potential side effects because they are less complex and better defined as compared to cell therapies.

The first report of native MSC-EV therapy in humans encompassed the treatment of one patient suffering from severe therapy-refractory acute graft-versus-host disease (GvHD) with EVs derived from four

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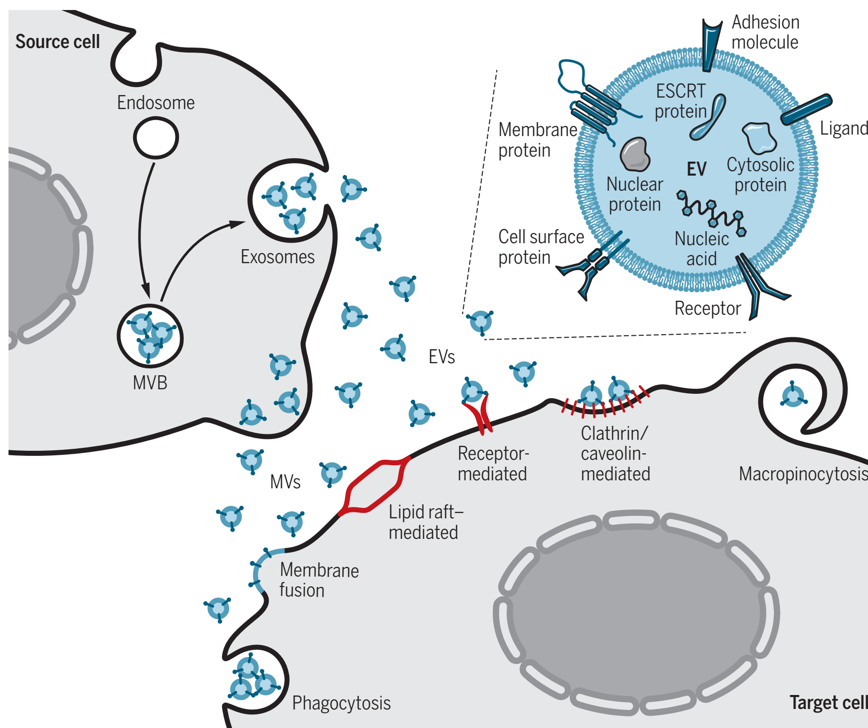

Fig. 1. EV biogenesis, general EV composition, and uptake. EVs are formed by two mechanisms. Exosomes are formed by the endocytic pathway through invagination of the endosomal membrane, which forms multivesicular bodies (MVBs) that can fuse with the plasma membrane to release exosomes into the extracellular milieu. Microvesicles (MVs) arise from the outward budding and fission of the plasma membrane. All subtypes of EVs share a general composition of an outer lipid bilayer and various proteins, lipids, and nucleic acids carried by the vesicles. The specific content of EVs is largely dependent on biogenesis, cell source, and culture conditions. EVs have been suggested to be internalized into target cells by various uptake mechanisms including membrane fusion (*171*) and different endocytic pathways including phagocytosis (*172*), receptor-mediated endocytosis (*173*), lipid raft–mediated endocytosis (*174*), clathrin-mediated endocytosis (*175*), caveolin-mediated endocytosis (*176*), and macropinocytosis (*176*).

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different bone marrow donors. The therapy was associated with improvement in clinical GvHD symptoms within the first week of MSC-EV therapy that remained stable 4 months after treatment (*66*). There have been few clinical studies conducted to date, which have evaluated native MSC-derived EVs. The first was a phase 1 clinical trial to evaluate human umbilical cord blood–derived MSC-EVs for the modulation of β cell mass in type 1 diabetes mellitus (ClinicalTrials.gov identifier NCT02138331); however, no information has been made available for this trial. The same team conducted a subsequent randomized, placebo-controlled, phase 2/3 clinical pilot study to investigate the safety and therapeutic efficacy of human cord blood–derived EVs in inhibiting the progression of grade III and IV chronic kidney disease (CKD). Outcomes showed that MSC-EV administration was safe, modulated the inflammatory immune reaction, and ameliorated overall kidney function in grade III-IV CKD patients (*67*). In addition, a phase 1 clinical trial to assess the safety and efficacy of MSCs and MSC-EVs for promoting healing of large and refractory macular holes (MHs) is currently ongoing (NCT03437759), and one further clinical trial is in the recruitment stage (NCT03437759). Although very early in terms of clinical use, together with evidence from preclinical studies, these clinical observations indicate that harnessing the innate ability of the MSC secretome by administration of one of the components, MSC-EVs, may hold promise for an acellular, off-the-shelf therapeutic strategy. An overview of clinical trials using EVs is shown in Table 2.

IMPACT OF ISOLATION METHODS ON EV INTEGRITY AND PURITY

The current standard technique for EV isolation is differential centrifugation with ultracentrifugation, encompassing a series of centrifugations to remove floating cells and cellular debris, followed by ultracentrifugation to pellet EVs (*11*, *68*). This technique is limited by low EV recovery, risk of co-sedimentation of nonvesicular macromolecule contaminants, and disruption of EV integrity. Furthermore, ultracentrifugation is laborious and time consuming and has limited scalability. It is also associated with EV aggregation due to high gravitational forces (*69*, *70*). When performing ultracentrifugation on MSC-CM, a very large portion of soluble proteins also get pelleted, calling into question whether claims of EV therapeutic utility isolated by ultracentrifugation should solely be attributed to the EV fraction and not a combination of soluble proteins and EVs. To effectively reduce non–EV-associated protein contamination in the EV pellet after ultracentrifugation, a density gradient separation step, using, for instance, iodixanol, to separate the EVs based on their density can be used (*11*, *71*). However, if the medium from which the EVs are isolated is of more complex nature than cell culture CM, such as body fluids, then other contaminants with similar density, such as lipoprotein particles in blood plasma, will colocalize (*72*).

Size-based isolation techniques are being increasingly used for EV isolation. There is, however, overlap in the size of EVs generated by different biogenic mechanisms. Ultrafiltration devices (*73*) and TFF systems (*74*) can concentrate EVs. Subsequent size exclusion chromatography (SEC) can be used to purify the EVs from co-isolated contaminants. SEC separates small molecules by transiently trapping them in pores of a matrix, whereas larger particles flow through (*69*, *75*, *76*). Small commercial SEC columns are suitable for relatively small volumes, such as blood plasma, as opposed to large volumes of CM, and combining SEC with bind-elute chromatography and a filtration step can be used for scalable EV isolation (*77*). The addition of SEC has improved EV integrity, protein purity, and functionality compared to ultracentrifugation-based isolation (*69*, *78*).

Polymer-based precipitation methods including commercial isolation kits and PEG precipitation have also been adopted for EV isolation and applied in clinical settings primarily for biomarker assessment (*66*). These precipitation methods have been widely used and demonstrate high recovery of EVs; however, the purity is often rather poor with coprecipitation of nonvesicular-associated protein and nucleic acid contaminants that may confound conclusions of EV content and function (*79*).

Other techniques, such as affinity-based capture, use known EV composition properties. For instance, immunoaffinity capture by anti-EpCAM and anti-CD63 antibodies has been used for EV isolation

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Table 2. Clinical trials of EV-based therapies. CEA, carcinoembryonic antigen; GM-CSF, granulocyte-macrophage colony-stimulating factor; imDCs, immature DCs; mDCs, mature DCs; N/A, not applicable; siRNA, small interfering RNA.

Indication	Phase, patients	EV source	EV manipulation	Results/status
Melanoma (*54*)	Phase 1, $n = 15$	imDCs, autologous	Pulsed with peptides	Safe, well tolerated; 2 stable disease, 1 minor response, 1 partial response, 1 mixed response
Non–small cell lung cancer (*93*)	Phase 1, $n = 4$	imDCs, autologous	Pulsed with peptides	Safe, well tolerated; 4 stable disease (where 2 had initial progression)
Non–small cell lung cancer (*95*) [NCT01159288]	Phase 2, $n = 22$	mDCs, autologous	Pulsed with peptides	32% with stable disease, primary endpoint (>50%) not reached
Colon cancer (*105*)	Phase 1, $n = 40$	Ascites, autologous	± GM-CSF–induced CEA	Safe, well tolerated; 1 stable disease, 1 minor response (both in CEA group).
CKD (*67*)	Phase 2/3, $n = 40$	MSCs, allogeneic	Unmodified	Safe, well tolerated; improved kidney function (improved eGFR, s- creatinine, and b-urea); decreased inflammation (↑IL-10, ↑TGF-β1, ↓TNF-α)
Colon cancer [NCT01294072]	Phase 1, $n = 35$	Plant derived	Loaded with curcumin	Active, not recruiting
Radiation and chemotherapy induced oral mucositis [NCT01668849]	Phase 1, $n = 60$	Grape derived	Unmodified	Active, not recruiting
Type 1 diabetes [NCT02138331]	Phase 1, $n = 20$	MSCs, allogeneic	Unmodified	Unknown status
Malignant ascites and pleural effusion [NCT01854866]	Phase 2, $n = 30$	Tumor derived	Loaded with chemotherapeutic drugs	Unknown status
Malignant pleural effusion [NCT02657460]	Phase 2, $n = 90$	Malignant pleural effusion	Loaded with methotrexate	Recruiting
Ulcers (wound healing) [NCT02565264]	Phase 1, $n = 5$	Plasma, autologous	Unmodified	Recruiting
Acute ischemic stroke [NCT03384433]	Phase 1/2, $n = 5$	MSCs, allogeneic	Enriched by miR-124	Not yet recruiting
Insulin resistance and chronic inflammation in polycystic ovary syndrome [NCT03493984]	N/A	Plant derived (ginger and/or aloe)	Unmodified	Not yet recruiting
Metastatic pancreatic cancer [NCT03608631]	Phase 1, $n = 28$	MSCs, allogeneic	KrasG12D siRNA (iExosomes)	Not yet recruiting
MHs [NCT03437759]	Phase 1, $n = 44$	MSCs, allogeneic	Unmodified	Recruiting
Bronchopulmonary dysplasia [NCT03857841]	Phase 1, $n = 18$	MSCs	Not specified (UNEX-42)	Not yet recruiting

with high purity (*80*). The high specificity of immunoaffinity methods will, however, only capture antigen-positive EVs and is compromised by the heterogeneous expression of EV markers on different EV subpopulations (*81–83*). The EVs not expressing the targeted marker will thus not be captured. The capturing beads or antibodies may also interfere with downstream analysis. To overcome this later issue, another affinity-based approach targets phosphatidylserine, which is exposed on the surface of some EVs, through calcium-dependent binding to a transmembrane protein [T cell immunoglobulin and mucin domain protein 4 (Tim4)] decorated on magnetic beads. By adding calcium-chelating buffer, the captured EVs are released from the beads (*84*). More recently, magnetic isolation of EVs using lipid-based nanoprobes, which permits intact, purer EV isolates in a much shorter timeframe compared to ultracentrifugation, has been developed (*85*). For small-scale EV isolation and high-throughput screening, for example, body fluid samples for diagnostics, several different microfluidic techniques, referred to as lab-on-chip devices, including di-electrophoresis, immunoaffinity, hydrodynamic-based methods, and magnetic-based techniques, have been used (*86–88*).

Future clinical trials and transition to regulatory-approved clinical therapy will likely require not only a greater scalability of isolation,

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higher purity, retained integrity, and functionality but also clearly defined components, standard operating procedures for reproducibility, quality control criteria, and sterility (*14*). In addition, EV production should be carried out with defined medium conditions, devoid of xenogeneic substances and serum-derived vesicles, which otherwise have a high risk of contaminating the isolated EV sample (*89*). A combinational approach using the advantage of different isolation techniques, such as TFF in combination with SEC, might be optimal considering high scalability, reproducibility, and ability to be carried out in a closed system. Very recently, anion exchange chromatography (AIEX) was used to isolate EVs with comparable yield, EV marker presence, size, and morphology to those isolated by ultracentrifugation, with decreased protein contamination compared to TFF-purified EVs (*90*). Because AIEX EV isolation permits enrichment of EVs in a scalable manner, this technique also holds potential for translation to clinical use. In addition, the membrane signatures of EVs are being deciphered (*83*), which allows for further development of affinity-based isolation techniques to allow selection of EVs with desired properties. The large variety of isolation techniques available provides researchers the ability to use the isolation method most suitable for their application and downstream analysis (*9*).

EVs IN IMMUNOTHERAPY

The initial approach of EV-based therapies using the immunostimulatory properties of EVs to generate an antitumor effect showed efficacy in preclinical studies (*91*, *92*). Two phase 1 clinical trials using autologous DC-derived EVs (Dex) pulsed with tumor antigenic peptides for treatment of melanoma and non–small cell lung cancer, respectively, were conducted in 2005 (*54*, *93*) (Table 2). Both demonstrated feasibility and safety of EV administration given weekly over 4 weeks; however, the beneficial effects of the therapy were minor or nonexistent. Subsequent studies have demonstrated that the immunomodulatory effects of DC-derived EVs depend on the maturation state of DCs. imDC-derived EVs have been observed to be immunosuppressive, whereas mDC-derived EVs are used for their immunostimulatory properties (*94*). Consequently, a subsequent phase 2 study targeting non–small cell lung cancer (*95*) used interferon-γ (IFN-γ) stimulation to induce DC maturation and increase immune stimulation. Although the anticipated T cell activation response observed in preclinical studies was not seen in patients, increased NK cell activity was observed in some patients. The opposing features of imDCs and mDCs have been associated with different expression of major histocompatibility complex (MHC) I and II; costimulatory molecules such as CD40, CD80, CD86, and CD54; and the immunoregulatory molecule PD-L1 (*96*, *97*). PD-L1, which inhibits T cell activation, has been found on DC-EVs (*98*) and on tumor-derived EVs and is a mechanism for immune evasion by tumors (*99*).

The presence of PD-L1 on metastatic melanoma-derived EVs (in particular exosomes) has recently been shown to be a potential predictive marker of anti–PD-1 therapy response (*100*). PD-L1 expression on EVs led to the proposal of concomitant PD-L1 blockade with mDC-EVs (*95*). A recent study investigated the potential synergistic effect of Dex with or without a PD-1 antibody (PD-1 Ab) in addition to the U.S. Food and Drug Administration–approved inhibitor of several protein kinases, sorafenib, for the treatment of a mouse model of hepatocellular carcinoma (*101*). The rationale for this approach is based on the findings that hypoxia induced by sorafenib treatment leads to tumor immunosuppression through regulatory T cells, including increased expression of PD-L1. The authors found no differences in tumor size or survival when sorafenib, Dex, or PD-1 Ab was used as monotherapies or in dual combinations. However, the triple combination showed decreased tumor volume and prolonged survival.

Another recent study showed that different populations of imDC-EVs induced different types of T cell responses (*102*). Large imDC-EVs (pelleted at 2000*g*) induced prominent secretion of T helper 2 (T_H2)–associated cytokines, whereas CM (pelleted at 10,000*g*) and small imDC-EVs (pelleted at 100,000*g*, often referred to as exosomes) induced secretion of T_H1-associated cytokines. The authors showed that these different T_H1/T_H2 responses are associated with different ratios of EV surface T cell–binding proteins CD40, DC-SIGN (found on small and medium EVs), and CD80 (present on all EVs). In contrast, all EVs derived from IFN-γ–matured DCs, independent of size, displayed the antitumoral T_H1 immune response (*102*). Wahlund *et al.* (*103*) compared medium-sized EVs (termed microvesicles, pelleted at 10,000*g*) and small EVs (termed exosomes, pelleted at 100,000*g*) derived from ovalbumin (OVA)–pulsed DCs and found that only the small EVs induced an antigen-specific $CD8^+$ T cell response. Small EVs were also found to elicit higher antigen-specific immunoglobulin G (IgG) production compared to medium-sized EVs. In contrast to the previously mentioned publication, no differences in expression of MHC class I/II or the costimulatory molecules CD40, CD80, and CD86 were found between small- and medium-sized EVs (*103*). The greater immunostimulatory effect of small EVs was instead attributed to the presence of the OVA antigen, which was higher in small EVs compared to medium-sized EVs. The same research group has previously shown that the DC-EV–induced T cell responses are independent of EV MHC/peptide complexes when whole OVA antigen is present, using $MHCI^{-/-}$ mice (*104*). Although clinical trials with DC-EVs showed poor efficacy, the treatment was found to be safe and feasible, paving the way for the many ongoing clinical trials using EVs (Table 2). The more recent findings of the variable function of EVs depending on cellular state of the source cell and the combinational therapeutic strategies highlight important considerations for future clinical trials.

A phase 1 study used an alternative antitumor immunotherapy approach by isolating EVs from the patients' ascites fluid (Aex) (*105*). Patients suffering from colorectal cancer received Aex, with or without adjuvant treatment of GM-CSF, which previously had been found to induce increased antitumor immunity (*106*). Although the treatment seemed safe and was well tolerated, a treatment effect defined by a beneficial antitumor cytotoxic T lymphocyte (CTL) response was only observed in 2 of 20 patients receiving Aex and GM-CSF, and no response was found in the patients receiving only Aex. The completed clinical trials, as well as the numerous preclinical studies, indicate that immunostimulatory EV therapy is a feasible anticancer approach and that autologous EVs are safe and well tolerated.

EV-based vaccines against pathogens, using pathogen antigen-pulsed EVs, EVs from infected cells, and pathogen-derived EVs, have shown promising results (*107*–*111*). However, there is a potential risk of pathogen propagation associated with many of these approaches, and EVs have delivered functional viral RNA (*112*, *113*). Similar to eukaryotic cells, parasites, helminths, fungi, bacteria, and virus-infected cells release EVs (*114*). For instance, similar to eukaryote-derived EVs, bacterial outer membrane vesicles (OMVs) are released

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into the extracellular environment, are enclosed within a lipid bilayer, and carry bioactive proteins, lipids, nucleic acids, and virulence factors. OMVs are being assessed as vaccines in clinical trials. They are believed to offer an advantage over conventional vaccines and be effective against infectious diseases, such as tuberculosis and enteric diseases, which currently lack efficient treatments (*115*). Compared to other biological therapeutics, such as cell and virus therapies, EVs cannot divide and multiply, suggesting that EVs are safer from a tumorigenic and infectious perspective. However, there is a risk of co-isolating pathogens with EVs, such as viruses that can have similar biophysical properties to EVs. Viruses may also be internalized into EVs as a route of spreading and immune evasion (*116*). In addition, studies have reported EV-mediated transfer of oncogenic molecules from tumor cells to normal cells (*117*). More recent findings, however, indicate that oncogenic cargo in tumor EVs exerts a regulatory rather than transforming influence on normal cells (*118*). Preclinical and clinical observations thus indicate that EV-based vaccines, as antitumor or anti-pathogen treatment, are feasible and well tolerated but have yet to show consistent immunostimulatory therapeutic effect in humans.

EVs AS DRUG DELIVERY VEHICLES

EVs are being explored as natural delivery vectors for different cargos, including small molecules and drugs with suboptimal pharmaceutical properties, because they can transfer bioactive components across biological barriers. It is also possible to deliver proteins and different RNA species, such as siRNAs and microRNAs (miRNAs), which have been shown to have potent action once in contact with their mRNA targets but which may have low cellular uptake, suboptimal pharmacokinetics, off-target toxicity, or stability issues. Loading of cargo into EVs often requires manipulation of the EVs or the parental cells. The techniques of loading cargo into EVs can be divided into two basic approaches: exogenous loading (with incorporation of small molecules/proteins/RNA into or onto isolated EVs) and endogenous loading (providing cells with the means to incorporate small molecules/proteins/RNAs into EVs during their biogenesis). Exogenous modification occurs after EV collection, with the desired therapeutic cargo packaged into EVs by various manipulations including co-incubation (*119*), electroporation (*19*), and sonication (*120*). Alternatively, the cargo can be endogenously loaded by genetically modifying the parental cell to overexpress a desired RNA or protein of interest (with or without modification to promote packaging), which is then naturally incorporated into the secreted EVs for collection.

Various techniques have been explored to load isolated EVs with therapeutic cargo. Incubation of EVs with curcumin improved the bioavailability and anti-inflammatory effect of this drug in a mouse model of inflammation (*121*). Similarly, incubating EVs with the immunosuppressive miR-150 generated an miRNA-EV association that was functionally active (*122*). EVs as drug delivery systems have been explored for a variety of different small molecules, including curcumin, doxorubicin, and paclitaxel (*123*). Preclinical animal studies indicate enhanced potency of the EV–small molecule treatment with improved pharmacokinetic profiles including improved brain delivery and tumor penetrance, as well as efficient cargo delivery and retention in tumor cells, compared to other vehicles, such as liposomes and polymer-based synthetic nanoparticles (*123*). On the basis of these findings, clinical trials with curcumin or chemotherapeutic drug–loaded EVs are being conducted (Table 2).

An interesting improvement in incubation-mediated loading was demonstrated recently using hydrophobically modified siRNA (hsiRNA) for *Huntingtin* mRNA silencing, with a compelling and efficient effect in vitro and in vivo (*119*, *124*). Similarly, Gao *et al.* (*125*) recently demonstrated that an anchor peptide (CP05, identified by phage display) targeting CD63 on EVs could be used as a versatile tool for EV loading, showing efficient loading of functional targeting and therapeutic CP05-conjugated cargos on EVs. Delivery of CP05-conjugated dystrophin splice–switching phosphorodiamidate morpholino oligomer (PMO) in combination with a CP05-conjugated muscle-targeting peptide (M12) on EVs to dystrophin-deficient mdx mice resulted in restoration of dystrophin and phenotypic improvement. Another approach for EV loading uses electroporation to generate transient membrane pores to facilitate entrance of RNA species (*19*) or small molecules (*126*). Cargo loading into EVs has also been demonstrated by permeabilization using saponin, freeze-thaw cycles, sonication, and extrusion (*127*). Commercial cationic liposomes have been used for EV transfection; however, this approach is confounded by the inability to separate EVs and liposome micelles, with electroporation suggested to be a superior technique (*128*). The different exogenous loading techniques have pros and cons, and whether the cargo is loaded into or onto, or just co-isolated with EVs, is often difficult to determine. Furthermore, the loading efficiency seems to be quite variable. For instance, electroporation has been proposed to generate as high as 85% loading in some publications; however, no encapsulation efficiency was reported (*128*).

Others have reported very poor loading efficiency with electroporation (*129*), which has been explained by the formation of siRNA aggregates, often misinterpreted as siRNA-loaded EVs. Nevertheless, numerous publications have demonstrated successful cargo loading by electroporation, and differences between groups may be due to varying protocol conditions. Treatment with fibroblast-EVs, electroporated with siRNA and shRNA (short hairpin RNA)–targeting oncogenic mutant *KRAS* (termed "iExosomes") demonstrated cancer growth suppression and increased survival in several mouse models of pancreatic ductal adenocarcinoma (PDAC) (*20*). The authors electroporated 1 μg of RNA into 10^9 EVs and estimated that about 10% was left after a washing step; hence, about 10^8 EVs (equivalent to 0.15 to 0.2 μg of RNA) were injected intraperitoneally per mouse every second day. Tumor progression was suppressed during continuous EV treatment and lasted for another 10 days if the treatment was paused. Resumed continuous EV treatment at a more advanced disease state resulted in partial response with slower, but not completely suppressed, tumor growth.

More recently, the same group published clinical-grade production of bioreactor-cultured MSC-derived iExosomes (*130*). Similar to the initial publication, intraperitoneal injections with MSC-iExosomes increased the survival of mice with PDAC in several models. The iExosomes retained function after 5 months of storage at −80°C, indicating stability and clinical feasibility. Although repeated injections of EVs that target one of the main drivers of a malignant tumor may delay tumor growth, because most cancers have many driver mutations, it is not clear for how long a single assault on one of these mutant drivers could stave off tumor progression. The recently registered clinical trial using iExosomes to target metastatic pancreatic cancer (NCT03608631; Table 2) will hopefully generate further insight into the potential of EVs as drug delivery vehicles for RNA species.

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BIOENGINEERED EVs

In contrast to exogenous loading, endogenous loading implies that cargo is expressed in the producer cell to exploit the cellular machinery for cargo sorting into EVs. Similar techniques as used for exogenous EV loading, including incubation (*131*) and transfection/transduction (*132*, *133*), have been used to incorporate small RNA and small molecules endogenously into EVs via loading into producer cells. EVs have also been loaded by overexpression of mRNA/protein for a suicide gene, with the released EVs showing potency in a mouse model of schwannoma combined with systemic prodrug administration (*134*).

EVs can be further engineered by manipulating the parental cell to produce EVs with a desired trait. The pioneering publication by Alvarez-Erviti *et al.* (*19*) used engineered EVs for brain-targeted delivery of siRNA. To enhance the targeting properties of the EVs, a peptide obtained from the rabies viral glycoprotein (RVG) was introduced as a targeting peptide on the EV surface by transfecting the parental cells with a plasmid encoding Lamp2b, an EV membrane protein, fused to RVG. The parental cell was thus engineered to produce EVs with the targeting peptide on the surface, resulting in increased brain accumulation after intravenous injections. A more recent study used RVG-exosomes for miR-124 delivery to the infarction site in a mouse stroke model (*135*). Intravenous injection of these EVs after induced cerebral ischemia promoted neurogenesis. On the basis on this finding, a phase 1/2 clinical trial assessing MSC-derived EVs loaded with miR-124 as a treatment in acute ischemic stroke has now been registered (NCT03384433; Table 2). Another publication demonstrated increased tumor targeting and antitumor effects by engineered EVs loaded exogenously with doxorubicin (*126*). In this case, the EV source cell was engineered to express Lamp2b fused to αv integrin-specific iRGD (CRGDK/RGPD/EC) peptide, which previously had been demonstrated to have efficient tumor targeting properties when assessed in prostate, breast, cervical, and pancreatic cancer models (*136*). Similar engineering approaches have been used to display reporter moieties, such as *Gaussia* luciferase on EVs (*137*). Another study used the transmembrane domain of the platelet-derived growth factor (PDGF) receptor fusion to a ligand of epidermal growth factor receptor (EGFR) for the production of engineered EVs that displayed increased efficiency of antitumor miRNA delivery to EGFR-positive breast cancer cells (*132*). EV display of anti-EGFR nanobodies fused with glycosylphosphatidylinositol (GPI) anchor peptides, for sorting to GPI-rich lipid rafts in EV membranes, was demonstrated to enable increased binding of the nanobody-EV complex to EGFR-positive tumor cells (*138*).

In a more recent study, cholesterol-conjugated RNA aptamers were displayed onto EVs carrying siRNA as a targeted antitumor delivery modality (*139*). EVs loaded with survivin-targeting siRNA were engineered to display RNA aptamers targeting folate, prostate-specific membrane antigen, or EGFR to enhance binding to specific receptors overexpressed on cancer cells. The authors showed enhanced cancer cell targeting and tumor growth suppression in mouse models of colorectal, breast, and prostate cancer. Another engineering approach used optogenetically engineered EVs, which were successfully loaded with proteins of interest using a reversible protein-protein interaction module controlled by blue light (*140*). Moreover, Sterzenbach *et al.* (*141*) recently showed that a protein of interest could be sorted into EVs by exploiting the late-domain (L-domain) pathway. Proteins with L-domains, such as syntenin and Ndfip1, are involved in the biogenesis of MVBs and exosomes by taking part in the recruitment of components in the endosomal sorting complex required for transport (ESCRT) machinery (*141*). The authors tagged Cre recombinase with a WW tag, one of the smallest protein-protein interaction domains (*142*), that interacts with Ndfip1 through three L-domain motifs (PPxY), which led to sorting of WW-Cre into EVs. Functional delivery of WW-Cre by EVs was demonstrated by their ability to induce recombination in floxed reporter cells in vitro and in vivo (*141*).

A subtype of EVs, known as arrestin domain containing protein 1 (ARRDC1)–mediated microvesicles (ARMMs), was recently shown to deliver NOTCH receptors to recipient cells and induce NOTCH-specific gene expression (*143*). ARMMs can also be used for EV-mediated intracellular delivery of other macromolecules (*21*). Using chimeric proteins consisting of a protein of interest fused to ARRDC1, which drives the budding of ARMMs from the plasma membrane (*144*), the authors demonstrated functional delivery of the tumor suppressor p53 protein (ARRDC1-p53) in vivo. In addition, functional delivery in vitro of p53 mRNAs was shown, using two fusion constructs: (i) ARRDC1 fused to a short transactivator of transcription (Tat) peptide, which binds specifically to the stem-loop–containing transactivating response (TAR) element RNA, and (ii) TAR fused to p53 mRNA. Production cells, transfected with the two constructs, secreted ARMMs that delivered functional mRNA into recipient cells. The genome-editing CRISPR-Cas9/guide RNA complex was delivered via ARMMs to recipient cells by fusing Cas9 to WW-domains, which interact with the PPxY motifs of ARRDC1.

An alternative approach, implanted, engineered EV-producer cells termed EXOtic devices that overexpressed three candidate genes (STEAP3-SDC4-NadB), termed production booster, resulted in up to 15-fold increased EV yield (*145*). In addition to the booster construct, the producer cells were cotransfected with an mRNA packaging plasmid (L7Ae fused to the C terminus of CD63), an mRNA of interest with an inserted C/D_{box} into the 3′ untranslated region, which interacts with L7Ae of the mRNA packaging device, and a cytosolic delivery helper [constitutively active connexin 43 (Cx43 S368A)], as well as a targeting plasmid (such as RVG Lamp2b). The EXOtic devices attenuated neurotoxicity and neuroinflammation in vitro and in vivo in models of Parkinson's disease (PD) by delivery of catalase mRNA via EVs from implanted producer cells.

Engineered hybrid EVs are emerging as an alternative strategy for improved therapy delivery. Fusing EVs with synthetic liposomes modifies and tunes the exosomal interface to decrease immunogenicity, increase colloidal stability, and improve the half-life in circulation (*146*). Another new hybrid EV strategy was recently presented by Votteler *et al.* (*147*), where they introduce the concept of enveloped protein nanocages (EPNs). By incorporating a variety of synthetic proteins, EPNs, similar to EVs, use membrane binding, self-assembly, and ESCRT machinery proteins for their biogenesis. The EPNs efficiently delivered their content into the cytoplasm of target cells.

The many emerging engineering strategies for generating therapeutic EVs (Fig. 2) build on the increasing knowledge of EV biology including biogenesis as well as protein and RNA sorting into EVs. In addition to the above-described protein engineering, hijacking proteins involved in the packaging of proteins into EVs, such as Lamp2b, WW-domains, and ARRDC1, as well as RNA posttranscriptional modifications (*148*) and RNA binding proteins [for instance, hnRNPA2B1 (*149*) and SYNCRIP (*150*)] have been implicated in the sorting of small noncoding RNAs into EVs. These are potential candidates to use for controlled RNA packing into EVs.

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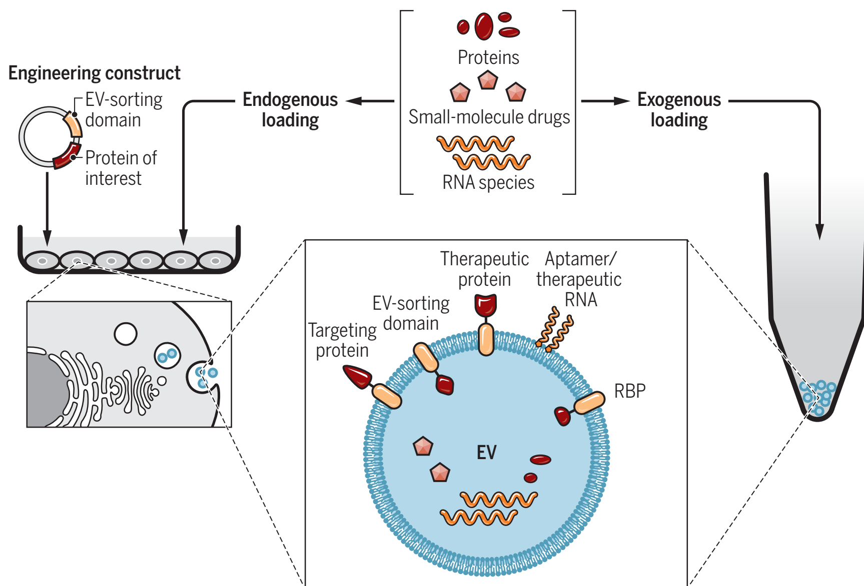

Fig. 2. EV engineering and loading strategies. EVs can be loaded with therapeutic entities such as RNA species, proteins, and small-molecule drugs through exogenous loading (loading of isolated EVs) or endogenous loading (loading during EV biogenesis). The producer cell can further be engineered to express EVs displaying therapeutic proteins or targeting peptides via chimeric proteins consisting of an EV-sorting domain fused to the protein of interest. Similarly, RNA-binding proteins (RBPs) can be explored to bind therapeutic RNA. RNA aptamers or therapeutic RNA can also be attached to EVs by hydrophobic modifications.

EVs AS THERAPEUTIC TOOLS TARGETING HEREDITARY DISEASES

EVs are emerging as useful platforms for the delivery of nucleic acids and proteins aimed at remedying genetic diseases. In this context, they have been used to transfer noncoding siRNA and miRNA targeting dominantly inherited diseases by inhibiting expression of the mutant allele via RNA interference (RNAi) and thereby altering the phenotype of recipient cells. EVs have also been used to transfer mRNA, proteins, and vectors targeting gene replacement in recessively inherited genetic diseases. Although therapeutic EVs may need to be administered at regular intervals if they supply a compound that has a relatively short half-life (such as drugs), EVs containing adeno-associated vectors (AAVs) can provide sustained transgene expression in nondividing cells in vivo. Important distinctions are whether the treatment aims to block a dominant gene defect, replace a missing gene, or modulate the downstream effects in genetic and nongenetic diseases.

Huntington's disease (HD) is a dominantly inherited neurodegenerative disorder caused by the aberrant expansion of CAG repeats in the *huntingtin* gene (*HTT*) (*151*) associated with altered miRNA expression (*152*) and characterized by cognitive impairment, progressive involuntary movements, and psychiatric changes (*153*). siRNAs incorporated into EVs have been used to target HD in disease models. hsiRNAs targeting both wild-type and mutant huntingtin mRNA were packaged into EVs exogenously. Addition of these EVs led to dose-dependent silencing of huntingtin mRNA and protein in primary cortical neurons in vitro, as well as silencing of *huntingtin* mRNA in the brain after infusion of EVs loaded with hsiRNAs into the cerebral spinal fluid in an HD mouse model (*119*). The same research group recently demonstrated further improvement of siRNA loading onto EVs by optimization of the siRNA-cholesterol chemistry (*124*). An alternate therapeutic strategy for HD, involving the delivery of exogenous miRNA, has also been explored (*154*). One mechanism involved in HD pathology includes alterations of transcriptional regulators, such as RE1-silencing transcription factor (REST), which is suppressed by miRNAs and sequestered by wild-type huntingtin under normal conditions. In HD, miR-124 is down-regulated, which increases the expression of its target REST mRNA (*152*). miR-124 was stably overexpressed in human embryonic kidney (HEK) 293T cells, and EVs released from them carrying elevated amounts of miR-124 were injected into the striatum of a mouse HD model. Although the therapeutic efficacy of these EVs was modest, without any behavioral improvement, a reduction in the expression of the target gene REST was observed (*154*). Together, these studies suggest that EVs incorporating either hsiRNA or miRNA are promising candidates for treatment of HD and other neurodegenerative disorders.

Neurofibromatosis (NF) is a group of dominantly inherited disorders caused by inheritance of one mutant allele of a tumor suppressor gene. Somatic mutation of the normal allele leads to tumor growth on the nerve sheath. NF type 2 is associated with growth of schwannomas, which are benign tumors derived from Schwann cells that form along the peripheral nerves, leading to compression of the nerves causing pain, weakness, paralysis, and hearing loss (*155*). Overexpression of the fusion prodrug-activating enzyme cytosine-deaminase::uracil phosphoribosyltransferase in HEK293T cells led to incorporation of this mRNA and protein into EVs that were then injected repeatedly into human schwannoma tumors grown in the sciatic nerve of nude mice (*134*). When this was combined with repeated systemic injection of the prodrug 5-fluorocytosine, which is converted to the chemotherapeutic agent 5-fluorouracil, the tumor regressed.

Cystic fibrosis (CF) is a recessive disorder caused by mutations in both alleles of the CF transmembrane conductance regulator (CFTR) gene, which causes deficient chloride channel activity and manifests as thick sticky mucus secretions, reducing the capacity of the lungs and increasing susceptibility to infection (*156*). Vituret *et al.* (*157*) collected EVs secreted from cells overexpressing CFTR and used these EVs to deliver CFTR-encoding mRNA and CFTR glycoprotein to CF patient cells in vitro. Chloride channel activity was restored in CF cells, and the maintenance of the effect was enabled by the newly synthesized CFTR proteins translated from exogenous CFTR mRNA. This in vitro study suggests a potential application of EV-mediated gene therapy targeting CF. EVs derived from lung MSCs reduce the inflammatory profile of CF cells in culture (*158*), but delivery of EVs to the affected cells in the lungs in vivo may be challenging.

PD is a neurodegenerative disorder that is characterized by elevated α-synuclein, brain inflammation, and secretion of reactive oxygen species (ROS), leading to death of dopaminergic neurons in the substantia nigra of the brain (*159*). Several causative genetic mutations have been identified for familial PD (*160*). Cooper *et al.* (*161*) achieved down-regulation of α-synuclein, the principal component

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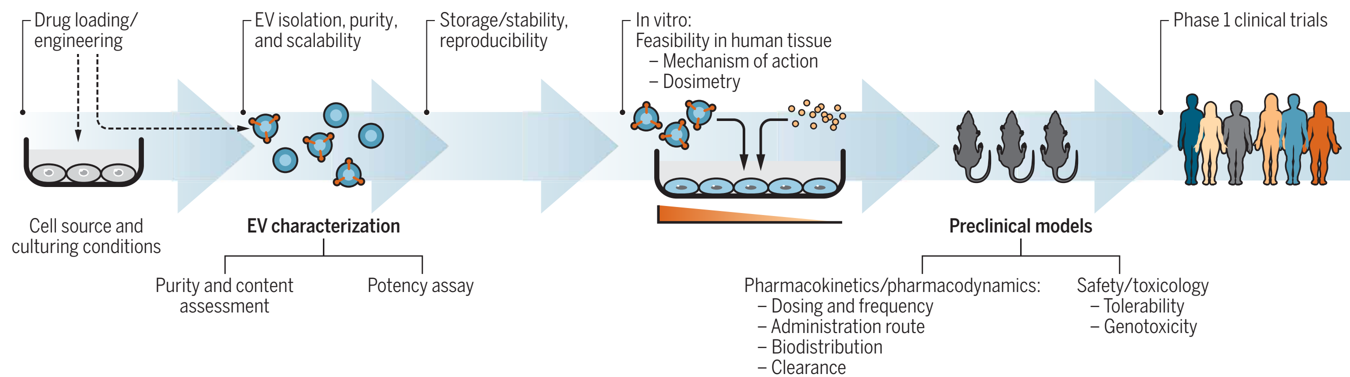

Fig. 3. Flowchart illustrating important considerations for developing EV therapeutics.

of filamentous Lewy bodies associated with PD pathology, in mouse brain after systemic injection of DC-EVs displaying RVG as a brain-targeting moiety and loaded with siRNA to α-synuclein (*161*). Catalase, a potent antioxidant, is diminished in PD patient brains, and therapeutic delivery of this protein to the brain is restricted by the BBB. Systemic administration of macrophages genetically modified to overexpress catalase, reduced inflammation, and provided neuroprotection in a mouse model of PD through the secretion of catalase-containing EVs by these macrophages (*162*). Subsequently, the same group harnessed EV therapy to overcome the BBB obstacle by loading catalase into EVs ex vivo (*127*) and then delivering them intranasally to the brain. This led to reduction of brain inflammation in a mouse model of PD, in contrast to free catalase administration, thus highlighting the potential of EV therapeutics for treating neurodegenerative disorders.

AAVs have emerged as an important gene therapy tool and have demonstrated promising results in clinical trials targeting a variety of genetic diseases (*163*). An AAV was the first clinically approved gene therapy product. Nevertheless, AAVs have certain limitations. Similar to other viral vectors, an intravenously administered AAV is primarily sequestered in the liver. Achieving therapeutic transgene expression often necessitates elevated vector doses and poses the risk of eliciting anti-capsid cytotoxic T cell responses (*164*). Furthermore, AAV administration carries the threat of triggering an immune response because humans often have preexisting neutralizing antibodies against AAVs (*165*). Critically, Maguire *et al.* (*166*) observed that AAVs associated with EVs (exo-AAVs) were superior to AAVs in transduction efficiency and ability to resist neutralizing anti-AAV antibodies in vitro and in vivo. It was possible to enhance exo-AAV transduction in the brain by displaying targeting peptides on the EV surface, and systemic injection of exo-AAV in mice led to more efficient gene delivery to the brain at low vector doses compared to conventional AAVs (*167*). Exo-AAV has since demonstrated therapeutic potential for the treatment of genetic blood disorders, specifically exhibiting lower susceptibility to neutralization by anti-AAV antibodies and efficient transduction of the liver at low vector doses in vivo with efficient correction of hemophilia B (*168*). More recently, exo-AAVs have been used to deliver transgenes to inner ear hair cells and consequently rescue hearing in a mouse model of hereditary deafness (*169*). Together, these studies demonstrate that although AAVs have immense promise for the treatment of genetic diseases, inefficient transgene expression in certain applications, coupled with immune response concerns, limits some of their applications. EVs can be harnessed to deliver AAVs and surmount some of the current AAV-associated clinical challenges, rendering exo-AAV a potent gene delivery system.

LOOKING FORWARD

Intense research within the field of EVs over the last decade has increased our understanding of the biogenesis, molecular content, and biological function of EVs. There are, however, hurdles yet to overcome before using EVs as therapies (Fig. 3). Choosing and characterizing an appropriate cell source for EV production according to the intended therapeutic use is of utmost importance. The well-studied MSCs and DCs are likely to be used, at least in certain disease settings, owing to their immunomodulatory properties and previous safe use in clinical settings. However, there is a growing list of additional cell sources requiring further characterization and testing, which could be suitable for clinical use, for example, endothelial progenitor cells for myocardial infarction (*51*) or amnion epithelial cells for treatment of lung fibrosis (*49*). Another aspect that needs to be addressed is the expansion format and culturing conditions of cells (2D/3D versus suspension culture, effects of culture media on yield, and composition of EVs). A variety of loading procedures and isolation methods are currently being developed and optimized for EV isolation, but identifying an optimal method that allows for scalable isolation of pure, clinical-grade EVs is still ongoing. To use EVs as off-the-shelf therapies, stability and storage must be further examined. In addition, the potency of the isolated EVs must be assessed in standardized potency assays, which are currently lacking. Therapeutic EVs must further be characterized in relevant preclinical models to assess safety/toxicology and the pharmacokinetic and pharmacodynamic profiles to support clinical dose predictions.

The multiple observations of the impact of EVs on various biological processes in the body and their ability to transfer bioactive components over biological barriers, as a means of intercellular communication, suggest that EVs could be harnessed for use as therapeutic agents. Potential therapeutic approaches include using EVs as drug delivery vectors, immunomodulatory or regenerative therapies, and antitumor and pathogen vaccines. The completed and ongoing clinical trials (Table 2), as well as numerous preclinical studies (*19*, *20*, *125*, *170*), indicate that EV therapy is feasible and that EVs are safe and well tolerated.

EVs are emerging as highly potent therapeutic entities, highlighting the therapeutic potential of the innate properties of EVs and use as a system for small-molecule drugs, RNA species, and therapeutic protein delivery in combination with targeting moieties. EVs

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represent a sweet spot between drug delivery, biologics, and cell therapies: They can be used as nature's own delivery tool, act as biotherapeutics, and mimic the action of cellular therapies without the drawback of proliferation. The field is still in its infancy, but research to optimize EV production and to dissect the complex EV biology, content, and function is ongoing. It seems likely that EVs will become a future platform of highly potent multifaceted biopharmaceuticals.

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Oscar P. B. Wiklander, Meadhbh Á. Brennan, Jan Lötvall, Xandra O. Breakefield and Samir EL Andaloussi

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