Review Article

Stem Cell INVESTIGATION

Engineering of extracellular vesicles as drug delivery vehicles

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Abstract: Extracellular vesicles (EVs) are secreted membrane-enclosed nano-sized particles (40–1,000 nm) that deliver biological information between cells. The molecular composition of these subcellular particles includes growth factor receptors, ligands adhesion proteins, mRNA, miRNAs, lncRNA and lipids that are derived from donor cells. A number of studies demonstrated that stem cell-derived EVs are the key mediator of tissue repair and regeneration in multiple animal disease models. In addition, the composition of these particles is known to be altered in cancer and disease pathology suggesting them for useful in diagnostic and therapeutic purposes. Their endogenous origin and biological properties offer benefits over conventional drug delivery systems (DDS), such as liposome, synthetic nanoparticles and prompted the further application of EVs as drug delivery vehicles for chemical drugs, genetic materials and proteins. The contents of EVs can be efficiently modified by chemical, biological or physical means. Thus, EVs can be an innovative DDS as it can overcome physical and biological barriers and safely deliver therapeutic drugs to target tissues. In this minireview, we summarized current progress on the strategies of drug loading onto EVs; *ex vivo* and *in vivo* loading.

Keywords: Extracellular vesicles (EVs); exosomes; microvesicles; drug loading; stem cells

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Introduction

A number of intercellular communication systems that deliver biological signals from cells to cells are discovered; these includes soluble factors (hormones, cytokines and growth factors and chemical messengers) and extracellular vesicles (EVs). First described by Trams *et al.* (1), EVs have been successfully isolated from different extracellular fluids including blood (2), urine (3), cerebrospinal fluid (4), breast milk (5), saliva (6) and even in culture supernatant (7).

They can be classified as exosomes, microvesicles (MV) and apoptotic bodies based on their biogenesis and/or size (8) (*Figure 1*). Exosomes, also known as nanosphere (size of 40 to 150 nm in diameter) are produced by invagination of endosomal membranes to form multivesicular bodies (MVBs) in endosomes and secreted by fusion of these vesicles with plasma membrane (9). Due to their subcellular origin, exosomes contain endosomal membrane proteins, membrane transport and fusion proteins (GTPases, Annexins and flotillin), tetraspanin proteins (CD63, CD81, CD82, CD53, and CD37), heat shock proteins, proteins associated with lipid rafts, including glycosylphosphatidylinositol-anchored proteins (10,11), and proteins involved in MVB biogenesis (Alix and TSG101) (12). Exosomes are rich in glycosphingolipids, cholesterol, phosphatidylserine and ceramide in the lipid bilayer (13) that accounts for their unique rigidity. Additional components of EVs include mRNAs, micro RNA (miRNA) and non-coding RNAs (14).

Microvesicles, also known as microparticles and ectosomes, are of 50 to 1,000 nm in diameter, produced

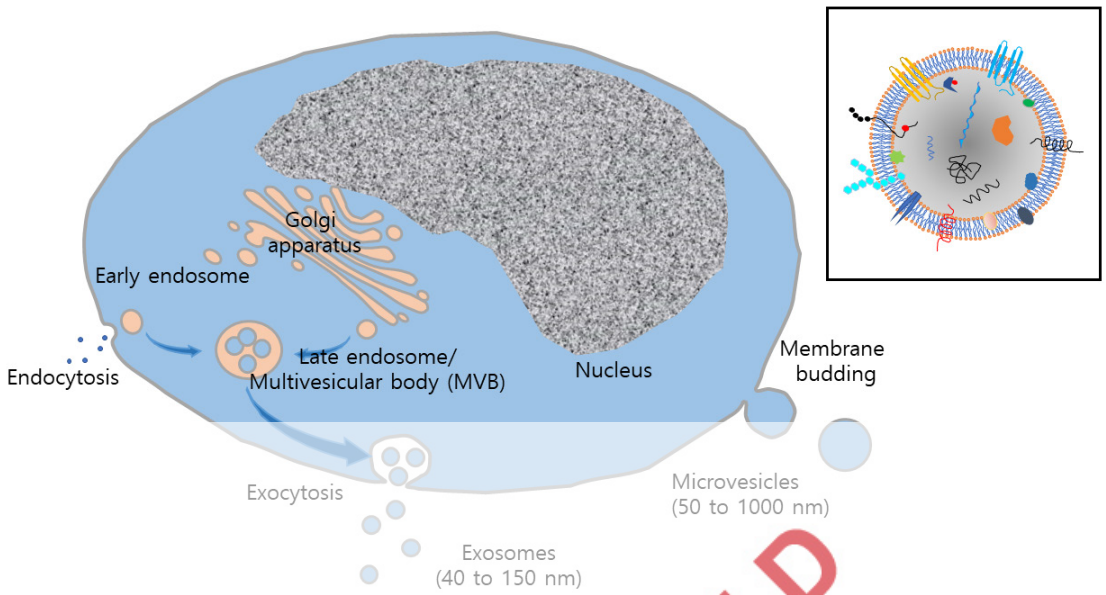

Figure 1 Formation of exosomes and microvesicles. Exosomes are secreted vesicles derived from intraluminal vesicles within multivesicular body (MVB), which, in turn, formed by invagination of endosomal membrane. Upon fusion of MVB with plasma membrane, the exosomes are released in the extracellular space. Microvesicles (MVs) are formed by budding of plasma membrane. These extracellular vesicles (EVs) carry proteins, RNAs and lipids that are derived from donor cells.

by direct budding of plasma membrane and thus carry membrane proteins and lipids of plasma membrane from donor cells (15). Although a number of proteins may be used as MV markers, definitive marker(s) for MVs has not been identified. MVs are rich in phosphatidylserine on the outer leaflet (16). The release of MV shares similarities with viral release in terms of structural features and outward budding process (17). Biological molecules of the cargo include proteins (enzymes, growth factors, growth factor receptors, cytokines and adhesion molecules) (15) and nucleic acids, (mRNA, miRNA and ncRNA) (18). There is no standardized protocol for MV isolation available, many researchers utilize combination of differential centrifugation with sucrose gradient ultracentrifugation, size exclusion chromatography or immunoaffinity column in order to exclude cellular debris, exosome fraction and other subcellular particles during isolation (19). In contrast to exosomes or MV that are derived from heathy cells, apoptotic bodies (also known as apoptotic blebs, or apoptotic bodies) are released from apoptotic cells. Apoptotic bodies have a size ranging from 500 to 5,000 nm and contain subcellular contents (organelles), deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and histone proteins. Currently, major research interest in EVs for drug delivery is focused primarily on exosomes and MV.

EVs are produced by virtually all species of living organisms and carrying biological signals that can influence behaviors of recipient cells implying their important roles in the development and function of tissues and organs. Unlike soluble factors, membrane-bound EVs can carry multiple biological information at once including proteins, nucleic acids (14,20) and lipids that can be shared between cells. Once secreted from donor cells, circulating EVs via biological fluids can be taken up by local or distant target cells and deliver the contents of the vesicles to target cells. While exosomes and/or MV can be isolated by single or combinations of different methods, including sedimentation by ultracentrifugation, density gradient ultracentrifugation, antibody-based separation, ultrafiltration, size exclusion chromatography, high performance liquid chromatography and fluorescent-activated cell sorter (21,22), a clear distinction of exosomes from MV in regards to their size, morphology, density, composition and physiological function is a challenging project; therefore, we will refer to EVs as a collective terms for both exosomes and MV. This review will primarily focus on the issues related to the loading methods of therapeutic molecules into EVs for their clinical applications.

Physiological function of EVs

The ubiquitous presence of EVs in biological fluids and from almost all cell types in culture suggests a fundamental physiological function for these particles. While EVs may have different functions depending on their cellular origin, much attention was given to their role in tissue repair and regeneration. Tissue reparative potential of stem cells, mesenchymal stem cells (MSCs) in particular, have been extensively studied for last several decades (23,24). Recent studies revealed that most, if not all, of the tissue reparative activities are attributed to secretome of stem cells rather than cellular engraftment and integration (25,26). With its anti-inflammation/immunomodulatory, anti-fibrotic, anti-apoptotic, pro-angiogenetic activities and endogenous stem cell mobilization capacities, stem cell secretome became an ideal cell-free therapeutic strategy in a number of animal models, including myocardial infarct (27), liver diseases (28,29) and acute kidney injury (30). In particular, Lai *et al.* (31) showed that exosomes from conditioned medium of MSCs are the therapeutic entity for cardioprotection observed in their previous study (27). Their proposed roles in tissue homeostasis prompted their application in regenerative medicine. EVs from MSCs and other stem/progenitor cells demonstrated the tissue regenerative potential against acute and chronic kidney injury (32), heart muscle tissue after ischemic injuries of chronic myocardial infarction (33) and liver fibrosis (34). The diverse effects are from anti-fibrotic, inflammatory and proangiogenic activity of EVs. MSC-derived promoted functional recovery and neurovascular plasticity in animal model of ischemic stroke (35,36) via cytoprotective, anti-inflammatory, proangiogenic, anti-fibrotic and regenerative effects recapitulating the effects of EV donor cells. Because of their production ability, immune modulating capacity and clinical applicability, MSCs are the most preferred donor cell type (37). Yet, their innate angiogenic and tumor tissue homing characters, utilization of unmodified, but not chemical drug-loaded, EVs is not recommended in cancer therapy (38,39)

Unlike other drug delivery systems (DDS), EVs from stem/progenitor cells are considered to possess negligible immunogenicity as of their donor cells that lack MHC class II and co-stimulatory molecules (CD80, CD86 or CD40) (40). EVs from stem/progenitor cells are known to recapitulate immunosuppressive activity of their donor cells (41-43). In addition, EVs from cancer are the key contributor to tumor progression, metastasis and tumor-induced immune suppression (44). A recent study showed that allogeneic EVs from cardiosphere do not induce significant immune responses upon repeated subcutaneous injections [Mirotsou M, Blusztanj A, Tremmel I, *et al. Repeated doses of cardiosphere-derived cell extracellular vesicles are hypo-immunogenic. J Extracell Vesicles*, Proceedings of the Abstracts from the 4th International Meeting of ISEV (ISEV'15), 2015] as of the parental cells in a clinical study (45) suggesting that allogeneic EVs can be suitable for clinical applications.

On the contrary, EVs from professional antigen presenting cells, such as dendritic cells, B cells and macrophages are known to express functional immune modulating proteins including MHC-class I and/or MHC class-II (46-48) and EVs from these cells preferentially induce Th1-type (cell-mediated) immune response that directs T cells to attack abnormal cells (such as cancer cells) or cells infected with intracellular parasites (49). Yet, EVs from immature or regulatory DCs are known to exert immunosuppressive activities via inducing antigen-specific regulatory T cell activation in animal models of allograft tissue tolerance (50,51) or autoimmune diseases (52,53). Thus, care must be taken in selecting EVs from different cellular origins that is suitable for its intended use.

Although the identity of therapeutic factors in EVs are under intense debate, it is clear that RNA species (mRNA, miRNA and lncRNAs) in EVs are functionally transferred to recipient cells (14,54) and modulate the behavior of target cells. In addition to their tissue regenerative potential, researchers are exploring these particles as potential diagnostic tools (55,56) or delivery vehicles for therapeutic molecules (57,58).

EVs as drug delivery vehicles

Among the innovative drug delivery technology developed, EVs holds a great deal of promise for targeted drug delivery. While synthetic nanoparticles, liposomes and recombinant viral vectors have been exploited as therapeutic vehicles, even with extensive modification and formulation, their toxicity, bioavailability and target delivery were the key issues. For example, chemical modification (such as PEGylation or chitosan) of nanoparticles or liposomes can efficiently increase their systemic bioavailability while interfering their interaction with target cells thereby reducing their biodistribution in target tissues (59-61). Furthermore, these changes significantly increase the immunogenicity and induce immune response against the carriers thereby increasing their clearance upon subsequent

injection (62,63). In this regard, nano-sized natural EVs represent an excellent alternative for drug delivery. As the composition of EV's membrane is from donor cell (stem cells), these particles are non-immunogenic in nature allowing them to resist to fast clearance from circulation and thereby increasing the drug delivery efficiency to target tissues (64,65). As of their cellular counterparts, EVs are known to possess specific cell tropism or homing ability (66,67) by cell type specific proteins (with their surface ligand and adhesion molecules), one of the key requirements for targeted drug delivery.

Over 98% of potent drugs for central nervous system failed to exhibit meaningful activity in the brain and many show poor penetration of the blood brain barrier (BBB) (68). In this regard, EVs are the ideal DDS for BBB, as these particles are known to possess an ability to cross biological barriers and deliver proteins, RNAs, DNA and chemical drugs. Thus, EVs possess advantages of both synthetic drug carriers and cell-mediated therapeutics, while avoiding the inherent limitations associated with synthetic carriers and cellular therapeutics. In addition, EVs can be formulate to exhibit intended drug carrying activity through various approaches including biological, chemical and physical means. Encapsulation of drugs (chemicals, RNAs, DNA, proteins or lipids) into EVs can greatly increase their bioavailability by preserving their integrity and biological activity *in vivo*. Lipid membrane from donor cells are suited to avoid phagocytosis, degradation and modification in host circulation. In addition, these natural products of our body typically avoid entrapment in reticuloendothelial system (also known as mononuclear phagocytic system) and non-immunogenic in most, if not all, parameters. Hence, extensive studies are being explored this natural product for the delivery of drugs (therapeutic chemicals, nucleic acids and proteins).

Various approaches can be utilized for loading of therapeutic agents into EVs. These include (I) loading drugs (of chemical, proteins or genetic materials) to purified EVs *ex vivo*, (II) pre-loading of drug or therapeutic factors to donor cells prior to EV purification (*Figure 2*).

Ex vivo drug loading to purified EVs

Ex vivo loading strategies mostly utilize passive packaging of therapeutic molecules, ranging from simple incubation to more sophisticated chemical and/or physical methods. Hydrophobic (i.e., lipophilic) molecules, such as anti-oxidants, anti-cancer drugs, lipophilic dyes, can be spontaneously packaged into EV under ambient conditions. Indeed, successful loading of curcumin (69), doxorubicin (70) and paclitaxel (71) into EVs were demonstrated. Extensive studies have been done in Curcumin, a natural polyphenol with strong anti-inflammatory property (57). Curcumin was incorporated into EVs by mixing to enhance the bioavailability and effectiveness of this hydrophobic chemical. Compared to standard liposomes composed of phosphatidylcholine and cholesterol, EVs exhibit higher loading efficiency and loading capacity to hydrophobic chemical drugs (72). The desired functional capacities of EVs isolated by sucrose gradient centrifugation were observed in suppression of macrophage activation *in vitro* and in lipopolysaccharides (LPS)-induced septic shock animal model *in vivo*. Of particular interest to the field of EVs as DDS is cancer therapy. The elegant study of Yang *et al.* (73) provided evidence that EVs possess the ability to deliver drugs across the BBB where exosomes isolated from brain tumor cell lines (U-87, PFSK-1, A-712 and bEND.3) loaded with rhodamine 123 and paclitaxel or doxorubicin could be detected in the brain of zebrafish embryos. Chemical drugs can also be loaded into EVs by electroporation (58). Studies demonstrated the enhanced efficacy with decreased adverse effects typically associated with chemotherapeutic drugs when compared to either EV-free drugs or drug-loaded liposomes (74,75). Yet, the efficiency of drug loading by these methods is less than expected, possibly due to the cargo capacity of purified EVs that carrying numerous cellular proteins and RNAs in it. For example, drug loading efficiency for paclitaxel and doxorubicin was 7.2% and 11.7%, respectively, determined by HPLC (73). In addition, the accurate measurement of EV-loaded drugs is not easy and their relative activity can only be referred to the protein contents in the complex.

EVs are the natural carriers of various nucleic acids including mRNA, miRNA and various noncoding RNAs (76,77) and thus represent ideal vehicles for nucleic acid transfer. Although siRNA is effective means for the regulation of genes of interests, their low stability and transducibility in circulation dictates the necessity of vehicles that can protect and deliver these therapeutic molecules to target cells and tissues. For *ex vivo* transfer of genetic materials to EVs, electroporation was firstly introduced in siRNA loading into exosomes derived from dendritic cells (58). Electrical field was applied to create pores in the membrane of EVs temporally, thereby allowing the movement of siRNA into the lumen of EVs. The delivery of and selective silencing of target genes by siRNA

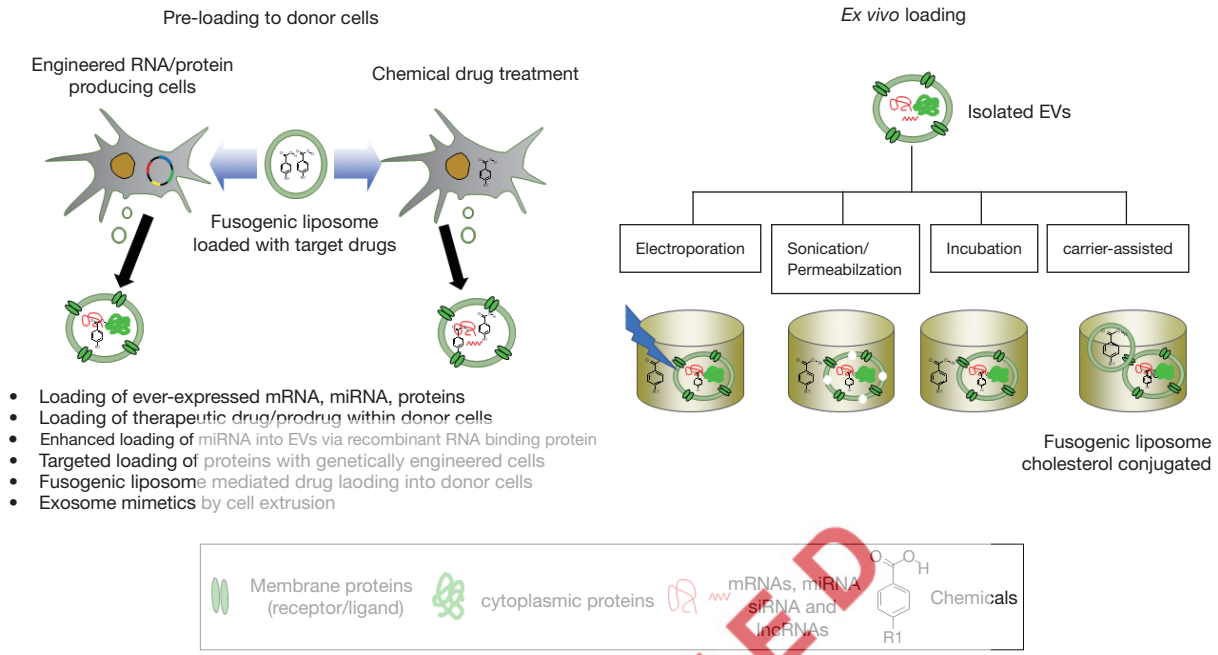

Figure 2 Different approaches to load therapeutic molecules into EVs. EVs can be loaded with chemical drugs, proteins and/or RNAs *ex vivo* and *in vivo*. The in vivo approach involves either transfection of genes (for protein products, mRNA, miRNA or lncRNAs) or pre-loading of chemical drugs in the donor cells, which are eventually loaded into EVs within the cells. The ex vivo approach involves the loading of therapeutics into isolated or purified EVs from donor cells. This can be done by various methods such as simple incubation, electroporation, freeze thawing, sonication and carrier-assisted delivery.

loaded EVs has been validated in a number of studies (78,79). Although loading efficiency of siRNA into EVs in these studies were up to 25% as determined by fluorescence spectroscopy of labeled siRNA (58,78), the actual loading efficiency in electroporation measured by Nanoparticle tracking analysis and confocal microscopy appears to be far less efficient than reported (80). Electroporation is also known to induce vesicular aggregation thereby affecting the integrity of the vesicles. While electroporation can be a suitable method for clinical setting as it can be easily controlled, several parameters including EV sources and concentrations, the cargo molecules (miRNA, mRNA, siRNA, lncRNA or plasmids) and the applying voltage with time for electroporation are difficult to standardize for optimal loading of the therapeutic cargo. The loading efficiency and capacity of exogenous DNA appears to be dependent on DNA size (linear DNA less than 1 kb is more efficient than large nucleic acids (81). In an effort to overcome this limitation, Wahlgren *et al.* (78) proposed a strategy to increase the incorporation of exogenous nucleic acids to EVs by utilizing pre-complexation of cationic liposome or micelle with siRNA and subsequent fusion with EVs that naturally carry a negative charge. Although two studies utilized chemical transfection reagents for siRNA loading into EVs, the efficiency could not be quantified and controlled (78,82) and making them not suitable for the clinical translation. To circumvent the utilization of harsh chemical or physical insults that may compromise the integrity of EVs, Didiot *et al.* (83) developed a robust and scalable methods for loading therapeutic RNA into EVs. The hydrophobically modified siRNA targeting Huntington RNA were efficiently loaded into EVs without altering the size and integrity of EVs. The silencing of Huntington mRNA by EVs was demonstrated *in vitro* using mouse primary cortical neurons as well as *in vivo* upon infusion into mouse striatum. Cholesterol-conjugation of siRNA is an efficient and reproducible method for siRNA loading into EVs (84). Sonication can be a suitable alternative for active loading of siRNA with minimal aggregation and degradation (85). With these approaches, the manufacturing processes for EVs loaded with exogenous nucleic acids can be scaled up for clinical uses with controllable loading

efficiency.

Aside from nucleic acids, EVs are a natural carrier of proteins (15). Since proteins cannot penetrate EV membrane freely, loading of EVs with proteins is a most challenging as it utilizes tactics to destabilize lipid bilayers by means of sonication, permeabilization, fusogenic liposomes, polymeric carriers and other physical insults. Haney *et al.* (86) reported that *ex vivo* catalase loading into EVs using various methods for Parkinson's disease therapy; the incubation at room temperature, permeabilization with saponin, freeze-thaw cycles, sonication, or extrusion. The study showed that a sonication and extrusion, or permeabilization with saponin resulted in stable EV reformation with high loading efficiency (18–26% measured by enzymatic activity), sustained release, preservation against proteases degradation and targeted delivery of this 240 kD protein *in vitro* and *in vivo*. Intranasal delivery of catalase-loaded EVs provided significant anti-inflammatory and neuroprotective effects with behavioral recovery in 6-OHDA treated mouse model of Parkinson's disease demonstrating the ability of EVs to deliver therapeutic proteins across BBB for the treatment of various neurodegenerative disorders. However, this protein loading method based on mechanical dispersion of EVs as well as protein denaturation or destabilization may significantly limit its applicability in clinics for unstable proteins.

Pre-loading of drugs to donor cells

Therapeutic agents such as chemical drugs can be incorporated to EVs from host cells. Pascucci *et al.* (71) demonstrated that EVs isolated from chemical drug-treated MSCs exhibited anti-proliferative activity to cancer cells *in vitro*. In another study, Lv *et al.* (87) reported that EVs isolated from cancer cells treated with different chemical drugs, such as paclitaxel, carboplatin, etoposide and irinotecan, exhibited strong anti-proliferative activity to cancer cells as well as NK stimulatory activity *in vitro*. Although these studies elegantly demonstrated the successful drug loading of EV *in vivo*, the pitfall of this approach is an inability to control the loading efficiency. Jang *et al.* (74) developed an efficient method to generate large quantity of exosome-mimetic nanovesicles by breakdown of drug-loaded donor cells (monocytes/macrophages) that exhibit efficient antitumor activity *in vivo*. In the same study, the authors demonstrated that removal of membrane proteins by trypsinization eliminated the therapeutic activity of the engineered EVs *in vitro* as well as *in vivo* implying the essential role of membrane proteins in tissue targeting and/or information transfer. More recently, Lee *et al.* (88) developed a liposome-mediated MV engineering for anticancer drug-loading where synthetic fusogenic liposomes loaded with chemical drugs (hydrophobic sensitizers as a model drug) were efficiently incorporated into host cell membrane and subsequently loaded into EVs.

Since EVs are the natural carriers of genetic materials, such as mRNA, miRNA and various noncoding RNA *in vivo*, the most preferred strategy is that utilizing EVs from donor cells transfected with therapeutic genes. Several studies harnessed the mechanism for generating EVs carrying high level of miRNA expressing vectors (89,90). EVs from miR-146b transfected MSCs effectively inhibited the glioma growth *in vitro* as well as in rat brain (90). EVs from miR-210 transfected neural progenitor cells (NPCs) protected endothelial cells from angiotensin II-induced oxidative stress (91). Alternatively, suppression of a miR-9, the key miRNA conferring chemoresistance of glioblastoma, can be achieved by EVs from anti-miR-transfected MSCs (92). Targeting of therapeutic miRNA-loaded EVs can be greatly enhance by utilization of the strategy in growth factor receptor-growth factor ligand interaction. Ohno *et al.* (89) showed that an efficient loading of let-7a miRNA to EVs and *in vivo* cancer targeting strategy by utilizing EGFR-binding EGF or GE11 domain on the surface of EVs. Monitoring with *in vivo* imaging system (IVIS) revealed that the XenoLight DiR-labeled GE11-positive EV localized mainly in the tumor at 24 h after intravenous injection, while little signal was detected in native exosome indicating the successful tumor targeting. The finding that sumoylated form of ubiquitously expressed RNA-binding protein, heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1), directs the sorting of miRNA loading into exosome through recognition of short sequence motifs over-represented in miRNAs (EXOmotifs) (93) provide a tool for efficient loading of selected regulatory miRNAs into EVs.

Since proteins are the major components of EVs, loading of recombinant therapeutic proteins expressed by host cells can be an attractive mode of drug delivery. A number of model proteins, including ovalbumin, catalase, glial cell-line derived neurotropic factor (GDNF) was successfully loaded into EVs from gene-modified host cells (94,95). Targeting EVs to specific tissues can be achieved by surface protein of EV membrane. Grapp *et al.* (96) demonstrated that intraventricular injection of EV expressing folate receptor-α on its surface can cross the BBB and deliver therapeutics

into brain parenchyma implying that utilization of these EV membrane proteins can greatly enhance the drug targeting to brain for treatment of neurodegenerative disease or malignancy. EVs from Epstein Barr virus-transformed or infected cells expressing gp350 protein can be used to transfer the contents to CD21 expressing B cells (97). Similarly, EVs from dendritic cells or exosome-mimetic nanovesicles from monocyte/macrophage expressing LFA-1 facilitate the interaction of EVs with activated T cells for delivering tumor antigens to induce tumor-specific immunity (98) or with tumor-associated endothelial cells for delivering chemotherapeutics to tumor cells (74), respectively. Studies have shown that tetraspanins, highly enriched in exosomes are known to participate in target cell binding via integrin binding (99,100). Thus, the delineation of tetraspanin web of EVs and the exploitation of these information to target selection will facilitate targeted drug delivery (101).

The genetic modification of donor cells can be used for targeting of EVs to designated tissues. Yang *et al.* (102) reported that systemic administration of EVs from dendritic cells engineered to express lysosomal-associated membrane protein 2 (LAMP2) that were electroporated with model siRNA against GAPDH or BACE1, a therapeutic target of Alzheimer's disease resulted in significant suppression of GAPDH or BACE1 in wild type mice. Similar study to Ohno *et al.* (89), tumor targeting of doxorubicin-loaded EVs from immature dendritic cells was greatly facilitated by engineering the fusion protein composed of LAMP2b with αV integrin-specific iRGD peptide (CRGDKGPDC) with less cardiac toxicity (70). Targeting of LAMP2b fusion proteins on the surface of EVs can be further enhanced by engineered glycosylation that protects the fusion proteins during biogenesis and secretion (103). For efficient targeting of therapeutic proteins that are not typically secreted or transported to plasma membrane, a recombinant DNA technology can be utilized. For example, a fusion gene encoding the vesicular targeting protein C1C2 and the model protein, ovalbumin, is engineered for ovalbumin loading into EVs (104). Yim *et al.* (105) recently developed a highly sophisticated method for intracellular delivery of target proteins during exosome biogenesis. They generated a cell line transfected with genes for two recombinant proteins, one for cryptochrome 2 (CRY2)-conjugated to target protein and the other for tetraspanin protein CD9 conjugated with CRY-interacting basic-helix-loop-helix 1 (CIB1) protein module. Since the photoreceptor cryptochrome 2 (CRY2) of *Arabidopsis thaliana* can bind to CIB1 by blue light wavelength, CRY2-conjugated cargo proteins can be actively and transiently docked into newly generated exosomes via CIB1 domain on CD9-CIB1 fusion proteins. Removal of blue light leads to detachment of target protein from CD9-CIB1 fusion proteins into the luminal space of exosomes. The potential of this strategy of protein delivery by exosome was further validated by transfer of Cre recombinase as a model protein *in vitro* as well as *in vivo*.

EVs can be engineered to deliver therapeutic mRNA/protein combination for cancer treatment. By transfecting donor cells (HEK-293T) with a vector construct consisting of cytosine deaminase (CD) fused to uracil phosphoribosyl transferase (UPRT), Mizrak *et al.* (106). generated MVs enriched with the suicide gene mRNA and protein. These studies validated that EVs may function as safe, efficient drug delivery vehicles to target tissues.

Therapeutic efficacy and toxicity of EVs are greatly influenced by their biodistribution (65). Studies on the various administration routes for efficient delivery of this therapeutic cargo to the lesions generated encouraging outcome (64,107). Of these, intravenous route is the most preferred route of administration. Like other DDS, systemic administration of EVs resulted in preferential accumulation in liver, kidney and spleen with rapid elimination in circulation. Multimodal imaging of systemically administered luciferase-loaded EVs *in vivo* revealed that the half-life of EVs were less than 30 min in most tissue and they were mostly cleared from the animals by 6 h (108). Similarly, a pharmacokinetic analysis revealed that the half-life of EV loaded with luciferase-lactadherin fusion protein from murine melanoma cells EV in the circulation is approximately 2 min and weakly detectable after 4 h indicating rapid clearance *in vivo* (109). Accumulation of EVs in the lung, liver, bone marrow and spleen was observed by sequential *in vivo* imaging suggesting that the distribution reflect the organotropism of murine melanoma during metastasis. These results are in sharp contrast to previous studies demonstrating that EVs can be detected in the liver and/or spleen, but not in circulation, at 24 h after systemic administration (89,110). The observed differences in biodistribution, tissue targeting efficiency and retention time in tissues may be attributed to the producing cells, vesicle sizes and the methods of EV preparation and thus warrant additional studies on the selection of optimal protocol for EV isolation, route of administration, formulation to increase their bioavailability and tissue targeting ability.

Conclusions

EVs clearly play key roles in normal physiology and diseases pathology. The use of drug-loaded EVs represent a next generation DDS with ability to transverse complex biological barriers such as BBB, while avoiding or overcoming a number of safety concerns related to drugs or vehicles, such as cytotoxicity, short biodistribution and low efficiency of targeted delivery. Chemical drugs and biological molecules (RNA, DNA and proteins) with low stability in circulation and/or low transducibility to target cells can be efficiently transferred to cytoplasm of target cells without undergoing endosomal and lysosomal degradation by this natural DDS. In addition, their composition and target specificity can be further tailored by engineering the producing cells or *in vitro* drug loading in accordance to target diseases or disorders; i.e., cancer therapy or tissue regeneration. A number of studies demonstrated that EVs may function as safe, efficient drug delivery vehicles to target tissues. Promising results of this cell-free therapeutics were obtained from a number of relevant animal models for human diseases and clinical translation of EVs has already initiated in cancer therapy and organ transplantation and their safety was validated.

However, the study of EVs is still in its early stages and a number of challenges remain to be addressed for their successful clinical translation. As shown by EVs from dendritic cells at different stages of maturation and MSCs, the heterogeneity in composition with functional activities of EVs is one of the key issues for their pharmaceutical acceptability. The choice of donor cells, culture conditions, refinement of methods for targeting and loading of therapeutic molecules must be explored in accordance to diseases. For clinical considerations of EVs, standardization of protocol for scalable production, isolation, storage and establishment of the criteria for quality control need to be established. Further study is required to better characterize the EVs and to delineate the underlying molecular mechanism responsible for their therapeutic effects and ultimately to exploit their full potential in the clinic.

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Footnote

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