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Extracellular vesicles: exosomes, microparticles, their parts, and their targets to enable their biomanufacturing and clinical applications

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Extracellular vesicles (EVs) are membrane vesicles, the submicron-size microparticles and the nanometer-size exosomes, that carry RNAs, proteins and lipids from their parent cells. EV generation takes place under cellular activation or stress. Cells use EVs to communicate with other cells by delivering signals through their content and surface proteins. Beyond diagnostic and discovery applications, EVs are excellent candidates for enabling safe and potent cell and gene therapies, especially those requiring strong target specificity. Here we examine EVs, their engineering and applications by dissecting mechanistic and engineering aspects of their components that endow them with their unique capabilities: their cargo and membranes proteins. Both EV cargo and membranes can be independently engineered and used for various applications. We review early efforts for their biomanufacturing.

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Introduction

Extracellular vesicles (EVs) are generated by most if not all mammalian cells [1–3] and carry RNAs, proteins, and lipids from their parent cells during EV generation, which takes place frequently under cellular activation or stress [2]. Among EVs, the submicron-size microparticles/microvesicles (MPs/MVs; also known as ectosomes) are the larger ones ranging from 100 to 1000 nm in size. They bud off the cytoplasmic membrane of the parent cell under normal physiological or pathophysiological conditions, including coagulation, inflammation, tumorigenesis, and differentiation [2]. Exosomes (Exos), distinct from MPs, are nano-size particle (<100 nm) which originate from multivesicular bodies through exocytosis [2,4]. Besides mammalian cells, outer membrane vesicles (OMVs), derived from bacteria (especially Gram-negative bacteria) are involved in stress response, promoting survival, pathogenesis, and interaction between bacteria in a community [5].

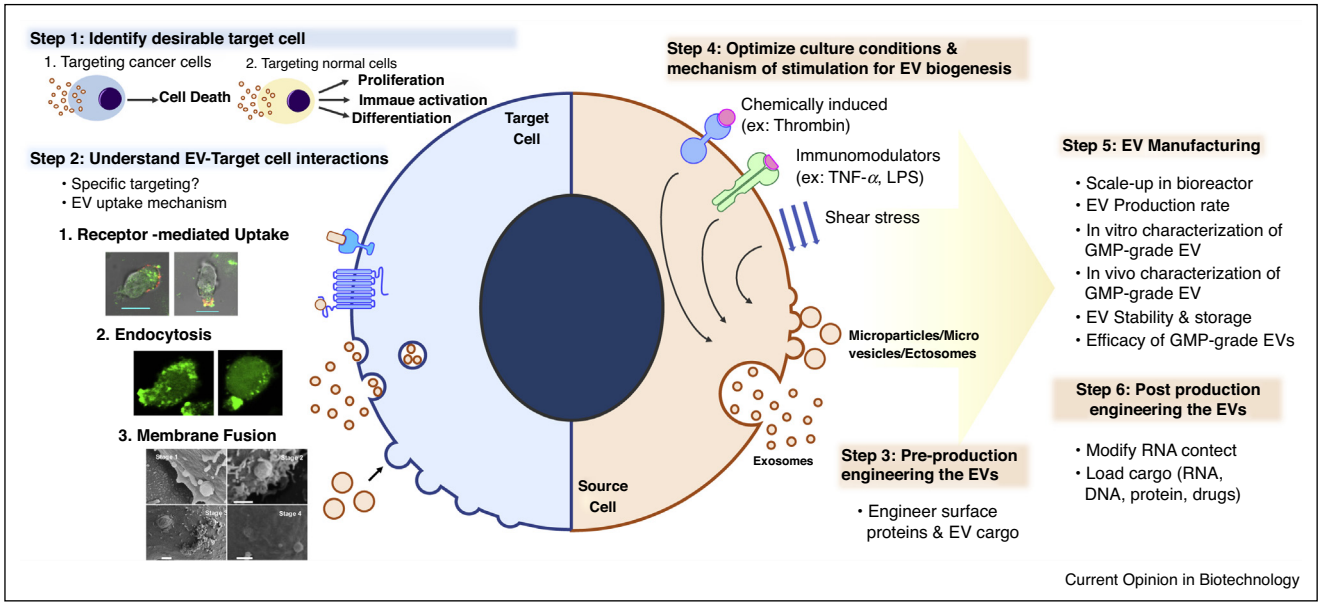
Cells use EVs to communicate with other cells by delivering signals through their content [2]. As reviewed (e.g. Refs. [6–8]), over the last few years, EVs have emerged as important mediators of intercellular communication regulating an ever-expanding range of biological processes, both on normophysiology and pathophysiology. The former includes enhancing and accelerating native developmental programs in immunology, vascular repair, and angiogenesis, while the latter includes carcinogenesis and cancer metastasis, neurodegenerative disorders, and infectious and cardiovascular diseases. As such, EVs are suitable for a broad range of applications, from minimally invasive diagnostic applications to therapeutic interventions, including cell therapies and macromolecular drug delivery. In order to pursue such applications involving EVs, better EV characterization, as well as better understanding of the mechanisms of cell targeting and methods for EV biomanufacturing are needed (Figure 1).

How do EVs recognize and deliver cargo to target cells?

Understanding how do EVs target and are taken up by cells is crucial for their applications [9,10]. The interaction typically starts with a ligand–receptor mediated binding, adhesion, or docking of EVs to target cells (Figure 1). The ligands and receptors involved are EV and target-cell specific, and, in some cases, this ligand–receptor recognition step is sufficient to alter the fate of target cells [11–13]. Yet, in most cases, EVs exert their biological effect through transferring of signaling molecules (miRNAs, mRNAs, proteins, phospholipids, or generally, a morphogen), which likely requires uptake of EVs by the target cells [2,14].

It has been suggested that the two major mechanisms used by targeted cells to take up EVs are endocytosis and membrane fusion [2,14] (Figure 1), and that cells use one

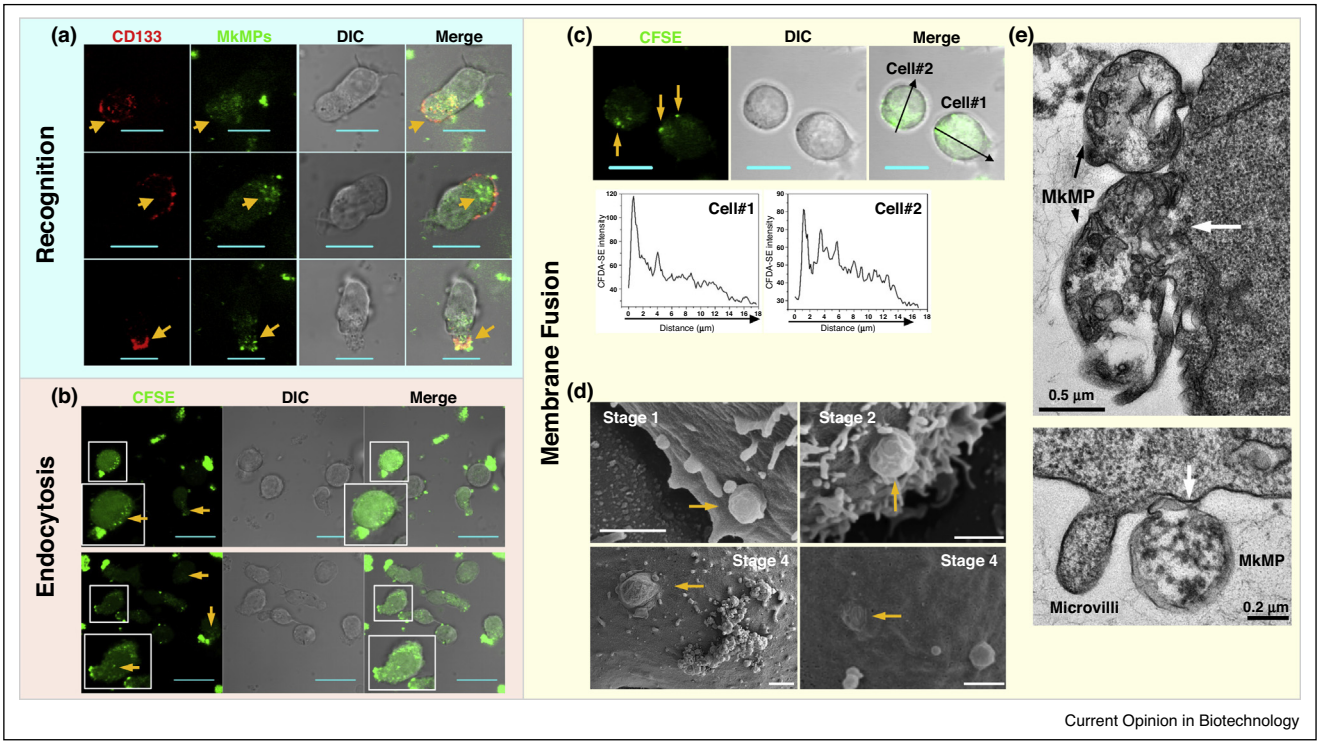
Figure 1



A systems view of EV-based applications.
Development for EV-based applications detailed in six steps. **Step 1:** Determination of the target cell type. One could target and induce tumor-cell death, or target normal cells to enhance cell proliferation, differentiation or trigger other positive phenotypic responses. **Step 2:** Identify one or more EV types for targeting the desirable cell type from Step 1, based on the biology of EV-to-target-cell interactions. Narrow down the options from EV's target specificity or biological outcomes. **Step 3:** Surface modification on EVs to enhance recognition of and uptake of EVs by target cells, if there are no naturally targeting EVs. **Step 4:** Determination and optimization of the method for EV biogenesis (chemically, physically, starvation-induced, stimulation-induced or other). **Step 5:** Manufacturing of EVs from bench to bioreactor scale, and optimization of EV purification and characterization for GMP-grade EVs. **Step 6**: Post-biomanufacturing modification of EVs to engineer membrane characteristic and content.

of these two mechanisms [15,16]. The mechanisms of EV uptake largely depend on the recipient cell type [17••]. Several endocytosis pathways have been examined in EV uptake. For example, macropinocytosis and clathrin-independent endocytosis dominate the uptake of EVs by tumor cells [18••], while phagocytosis is usually engaged by immune cells (dendritic cells, macrophages, or T cells) [19]. Receptor-mediated endocytosis such as clathrin-dependent or caveolin-dependent endocytosis was reported for microglia, macrophages or tumor cells [10,20]. Direct fusion of EVs with cells was also claimed in some studies [16,21], but the evidence for such fusion events was challenged [22••]. The fusion event requires the interaction among surface proteins from EVs and recipients cells, such as syncytin and its receptor Major Facilitator Superfamily Domain 2a (MFSD2a) [23]. EV uptake is examined in *in vitro* co-cultures of EVs with target cells. EVs are stained with a lipid-membrane dye (e.g. PKH26 or DiD) or a cytosolic dye (e.g. CFSE) for tracking the EVs and the cargo delivery into target cells. We have recently reported detailed studies on the uptake of megakaryocyte-derived MPs (MkMPs) by hematopoietic stem/progenitor cells (HSPCs) through both endocytosis and membrane fusion [22••] (Figure 2). We have used CFSE-stained MkMPs and identified individual intact MPs in target cells thus demonstrating MP uptake through endocytosis (Figure 2a and b) [22••]. To identify EV uptake via membrane fusion, transferring of fluorescence dye from the EVs to target cells were detected by confocal microscopy [16,21]. Using confocal fluorescent microscopy, we have shown the generation of gradients of CFSE dye carried by MkMPs into HSPCs, thus suggesting the delivery through membrane fusion (Figure 2c) [22••]. Electron microscopies can provide higher resolution and detailed imaging of EV uptake. With scanning electron microscopy (SEM), we have demonstrated the fusion of MkMPs to HSPCs in four stages (Figure 2d), with microvilli on the HSPC surface noted near the 'landing' site of MkMPs on the HSPCs [22••]. With transmission electron microscopy (TEM), fusion events of MkMPs into HSPCs were detailed based on different TEM textures of MkMPs compared to those of the target cells (Figure 2e), further supporting the likely role of microvilli on the HSPCs on the MP to target recognition [22••]. Further, we demonstrated that MkMPs recognize HSPCs through ligand–receptor interactions mediated by several surface antigens on MkMPs (CD54, CD11b, CD18 and CD43), and that the preferential site of MkMPs on the HSPCs is the uropod ('back tail') of the HSPCs (Figure 2a).

Figure 2



Mechanisms involved in EV target recognition and uptake by targeted cells based on the example of megakaryocytic MPs (MkMPs) targeting hematopoietic stem and progenitor cells (HSPCs).
(a) Confocal microscopy showing that MkMPs recognize and target HSPCs at the uropod area ($CD133^{+}$) of HSPCs. MkMPs were pre-stained with CFSE (green) dye before co-cultured with HSPCs for 3 hours. Scale bars represent 10 μm. Internalized MkMPs in HSPC uropods are indicated by yellow arrows. **(b)** Evidence for MkMP uptake by HSPCs via endocytotic processes. CFSE-stained MkMPs were co-cultured for 3–5 hours with day three cultured HSPCs. Intact MkMPs (yellow arrow) inside HSPCs were identified via confocal microscopy. Inserts amplify images to show more details. Scale bars represent 20 μm. **(c)** Evidence for MkMP fusion to HSPC membranes. CFSE-stained MkMPs were co-cultured for 3–5 hours with day 3 cultured HSPCs. Confocal microscopy images in the upper panel demonstrate CFSE dye gradients inside the cells emanating from one or few fluorescent particles (yellow arrow) on the cell surface. Scale bars represent 20 μm. Figures in the lower panel show the CFSE dye intensity profiles quantitating the dye gradient along the black arrows of cells #1 and #2. **(d)** MkMPs were co-cultured with HSPCs for 3–5 hours. Micrographs from scanning electron microscopy demonstrate four consecutive stages of the MkMP (yellow arrow) fusion into HSPC membranes. Scale bars represent 1 μm. **(e)** Evidence for MkMP fusion to HSPC membranes. MkMP were co-cultured with HSPCs for three–five hours. Micrographs from transmission electron microscopy display partial membrane fusion (white arrow). Adapted from the authors' own work (Ref. [22••]).

EVs alter the biology and fate of target cells through diverse mechanisms

Uptake of EVs enables delivery of EV cargo to recipient cells, thus triggering a broad spectrum of biological phenotypes. Tumor-derived EVs regulate the tumor microenvironment and impart an invasive effect in cancer progression and angiogenesis [24,25]. Exosomes derived from hepatocellular carcinoma cells delivered both SMAD Family Member 3 (SMAD3) protein and mRNA to circulating hepatocellular carcinoma cells, enhanced their adhesive ability, and supported their metastasis [26••]. EVs also play roles in cell differentiation and reprogramming. In our previous studies, MkMPs triggered megakaryocytic differentiation of HSPCs by transferring of RNA, especially miRNAs [22••,27]. Ultrasound-induced exosomes released from human dermal fibroblasts carried reprogramming factors and quickly induced cell differentiation of fibroblasts to neural-progenitor cells [28••]. MPs from endothelial progenitor cells activated angiogenesis by transferring of mRNA to other endothelial cells [29]. Delivery of miR-150 by monocyte-derived MPs to endothelial cells promoted angiogenesis [30]. EVs have also demonstrated therapeutic potential. For example, MPs derived from human bone marrow mesenchymal stem cells stimulated proliferation of tubular epithelial cells and protected cells from apoptosis through RNA delivery [31].

Translational applications of native EVs

As discussed, native EVs (MP and exosomes) have a good potential as therapeutic agents in translational applications. Below, we review the target specificity and the

Table 1

Translational applications of EVs

In vitro/in vivo	Functionality	Native cargo	Target cell	Ref.
Mesenchymal stem cell (MSC)-derived EVs				
In vitro	MSC-EVs targeted monocytes and B cells.	N/A	Monocytes, B cells	[33••]
In vitro	MSC-EVs increased the expression of anti-apoptotic genes (e.g.: Bcl-xL, Bcl2, and BIRC8) and decreased the expression of proapoptosis genes (e.g.: Caspase 1, Caspase 8, and lymphotoxin alpha) in human tubular epithelial cells.	N/A	Tubular epithelial cells	[34]
In vivo (xenograft tumor model)	MSC-EVs promoted cell growth of lung adenocarcinoma cancer cells.	miR-410	Lung adenocarcinoma cancer cells	[35]
Endothelial progenitor cell (EPC)-derived EVs				
In vitro and *in vivo* (SCID mice)	EPC-derived MVs were incorporated in endothelial cells by interaction with alpha-4 and beta-1 integrins expressed on the MV surface, and trigger angiogenesis, promoted endothelial cell survival, and proliferation.	mRNA	Endothelial cells	[29]
In vitro and *in vivo* (LPS-induced ALI in mice)	Administration of EPC-Exos ameliorated LPS-induced ALI and restored the *in vivo* pulmonary integrity. EPC-Exos enhanced the proliferation, migration and tube formation of endothelial cells (ECs).	miR-126	Endothelial cells	[36•]
In vitro and *in vivo* (murine sepsis model)	EPC exosomes treatment improved survival, suppressing lung and renal vascular leakage, and reducing liver and kidney dysfunction in septic mice.	miR-126-3p, miR-126-5p	HMVECs	[37]
In vitro and *in vivo* (AKI rat model)	EPC-MVs protected the kidney from ischemic acute injury by enhancing tubular cell proliferation, reduced apoptosis, and leukocyte infiltration, by delivering of miR-126 and miR-296. EPC-MVs specifically targeted endothelial cells and epithelial cells, but fibroblast-MVs did not.	miR-126, miR-296	Tubular endothelial cells, tubular epithelial cells	[38]
Megakaryocyte (Mk)-derived and platelet-derived EVs				
In vitro	Human megakaryocyte-derived microparticles induced megakaryocytic differentiation of hematopoietic stem/progenitor cells via transferring of RNA.	mRNA, miRNA	Hematopoietic stem/progenitor cells	[22••]
In vitro	P-EVs bound to neutrophil or endothelial cells through specific markers, and promoted the interaction between neutrophils and endothelial cells.	N/A	Neutrophils or endothelial cells	[41••]
Red blood cell-derived EVs				
In vitro	RBC-EVs bound to monocytes through CD11b/CD18 to activate endothelial cells.	–	Monocytes and granulocytes	[80]

Abbreviations: LPS, Lipopolysaccharide; ALI, Acute lung injury; AKI, Acute kidney injury.

nature of the native cargo of EVs derived from a few select cell types. These and additional reports are summarized in Table 1.

Mesenchymal stem/stromal cell (MSC)-derived EVs

MSC-derived EVs (MSC-EVs) have shown therapeutic effects in tissue repair based on their anti-apoptotic, anti-inflammatory, or anti-oxidant effects (reviewed in Ref. [32••]). MSC-EVs can enhance proliferation, and/or reduce apoptosis of epithelial cells in kidney disease, hepatocytes in liver diseases, or cardiomyocytes in heart disease, apparently by delivery of RNA or growth factors [32••]. Di Trapani *et al.* have shown that MSC-EVs specifically target monocytes and B cells, but not other lymphocytes (T, NK cells) in peripheral blood [33••]. Bruno *et al.* demonstrated specific anti-apoptotic effects from MSC-EVs, but not from fibroblast EVs, to human tubular epithelial cells by upregulating anti-apoptotic genes and downregulating pro-apoptotic genes [34]. In contrast, human umbilical cord MSC-EVs promoted cell growth of lung adenocarcinoma cells mediated by miR-410, thus suggesting another therapeutic option (miR-410 inhibition) to inhibit tumor progression [35].

Endothelial progenitor cell-derived EVs (EPC-EVs)

EPC-EVs target specifically endothelial cells or epithelial cells. *In vitro*, EPC exosomes (EPC-Exos) enhanced the

proliferation and migration of endothelial cells [36•]. *In vivo*, injection of EPC-Exos into mice alleviated Lipopolysaccharide(LPS)-induced acute lung injury (ALI) by transferring miR-126 to endothelial cells [36•]. EPC-Exos, which are enriched in miR-126-3p and miR-126-5p, improved cell survival in a murine sepsis model [37]. Deregibus *et al.* demonstrated that EPC microvesicles (EPC-MVs) specifically target endothelial cells via the surface proteins VLA-4 (alpha-4 and beta-1 integrins), thus triggering angiogenesis and enhancing endothelial survival [29]. In an acute kidney injury (AKI) rat model, EPC-MVs enhanced tubular endothelial/epithelial cells proliferation mediated by the delivery of miR-126 and miR-296 [38]. These EPC-MVs target endothelial/epithelial cells but not fibroblasts [38].

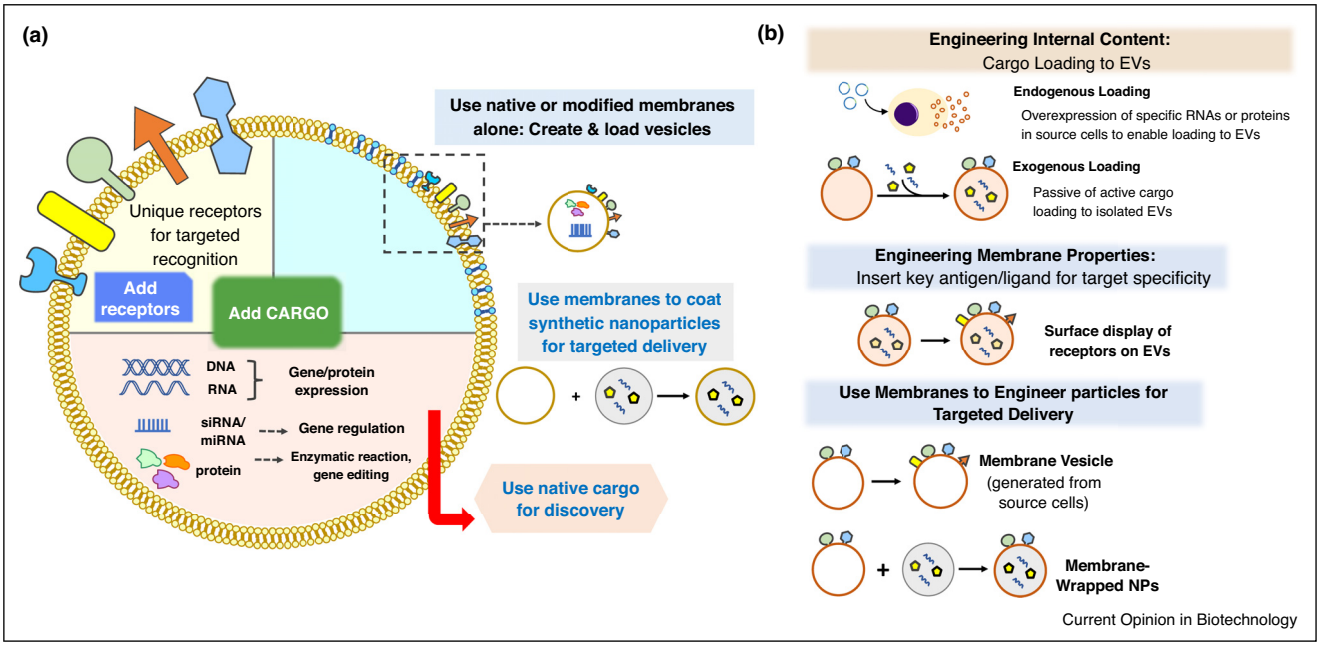
Megakaryocyte and platelet-derived EVs (Mk-EVs and P-EVs)

Mk-EVs and P-EVs are the most abundant EVs in circulation [39]. Although they have the same ontogeny and both present the megakaryocytic markers (CD41 or CD42b), they differ in that P-EVs are $CD62P^{+}$ while Mk-EVs (the most abundant in circulation of the two) are not. As discussed, Jiang *et al.* demonstrated that MkMPs target HSPCs specifically through membrane fusion and endocytosis, as mediated by surface proteins CD11b, CD18, and CD54 [22••]. MkMPs can transfer RNA [22••] to induce megakaryocytic differentiation of HSPCs [27]. P-EVs trigger angiogenesis and play a role in tissue repair and tumor progression [40]. In a recent study, P-EVs (mostly platelet-derived microparticles, PMPs) were shown to efficiently bind to neutrophils or endothelial cells to enhance the interaction between neutrophils and endothelial cells. CD62P or CD11b mediated the recognition of neutrophils by P-EVs, while CD61, CD21, and CD51 were involved in the interaction between P-EVs and endothelial cells [41••].

Engineering using EVs or their components for cargo delivery

EVs exhibit desirable native characteristics that makes them suitable as vehicles for cargo delivery (Figure 3). As summarized in Figure 3, all EVs components (their native cargo, surface proteins, and membranes), can be engineered for various applications. Loading of synthetic

Figure 3



Engineering inspired by EVs and their components: together and separately.
(a) The key components of EV include surface proteins (receptors), lipid-based bilayer membranes, and internal cargo (DNA, RNAs, small RNAs, and proteins). There are several possible technological applications based on EVs and/or their components. For example, native cargo can be used for discovery or diagnosis. **(b)** There are several engineering concepts to use EVs or their membranes for targeted delivery. For example, target specificity is mediated by unique native surface proteins, and can be enhanced by expressing additional antigens, ligands, receptors or antibodies. Additional cargo can be loaded into EVs for delivery to specific cell targets aiming to achieve various biological effects. Endogenous loading is achieved by overexpression of specific genes (via plasmid DNA loading) or directly loaded RNAs or proteins in EV-producing cells with transfecting of DNA vector. Exogenous loading is performed by passive (mixing) or active (electroporation, sonication, or extrusion) cargo lading to isolated EVs. Cellular membranes (native or modified) can be used independently to load cargo for targeted delivery. For example, membrane isolated from a specific cell type can be used to form membrane vehicles (MVs) carrying specific receptors for targeting. MVs can be used for exogenous cargo loading, or to wrap synthetic cargo-loaded nanoparticles.

cargo is an important first engineering goal. EVs can be also engineered to enhance target specificity. Finally, their native cargo can be used for diagnostic, discovery or therapeutic applications.

Cargo loading

Two main approaches have been used for cargo loading (Figure 3b). Endogenous loading was used for protein or RNA loading, whereby EV-producing cells were transfected with plasmid DNA [42] or RNA [43,44] to overexpress specific genes and/or proteins [45]. Also, rather than transfection, simple incubation of drugs with cells leads to drug loading upon EV biogenesis [46,47]. More investigations have focused on *exogenous cargo loading* largely through electroporation to load RNAs or drugs [46,48–50]. The loading capacity of cargo molecules varies among different EV subsets. Small RNAs (miRNAs [51] or siRNAs [52]) can be easily loaded into exosomes with electroporation. MVs/MPs, due to their larger size, can carry linear or plasmid DNA (pDNA either loaded by direct electroporation [50,53••] or transferred from the EV-producing cells [54]). Loading linear dsDNA larger than 1000 bp to HEK293T-derived exosomes was inefficient, while loading pDNA practically was impossible due to the small size of exosomes [50]. Both *in vitro* and *in vivo* cargo delivery by HEK293FT-derived exosomes and MPs demonstrated that functional protein expression was only possible from pDNA delivery [54]. We demonstrated that up to 4200 copies of pDNA can be loaded per MkMP, which is significantly higher than the exosome capacity for pDNA encapsulation (less than 10 copies per exosome) [53••]. For loading practically significant amounts of large size cargo molecules like pDNA, one must use MPs due to their higher cargo capacity [53••]. Other methods such as saponin-mediate loading, extrusion or dialysis have also been used for cargo loading into EVs, but electroporation was shown to be the most effective method [46].

Surface modifications

Surface proteins are essential for recognizing specific antigens on target cells (Figure 1). Beyond the natural target specificity of some EVs (Table 1), one could engineer EVs by expressing proteins or peptides on their surface to enable or enhance target specificity (Figure 3b). Ohno *et al.* engineered exosomes by expressing a peptide fusion protein to specifically target and deliver miRNA to breast cancer cells expressing EGFR (epidermal growth factor receptor [42]. Tian *et al.* demonstrated doxorubicin delivery by engineering dendritic-cell derived exosomes expressing the exosomal membrane protein Lamp2b-fused to an integrin-specific iRGD to target tumor cells *in vivo* [55]. In another application, using exosomes expressing Lamp2b fused to the neuron-specific RVG peptide, GAPDH-siRNA was specifically delivered to various cell types in the mouse brain [52].

Mechanisms of EV biogenesis

Key to the biomanufacturing process is the mechanism by which one can induce EV formation from various cell types. EVs are produced typically under physiological or pathophysiological stress or stimulation. Biogenesis of two major type of EVs (exosomes or MPs) is quite distinct. For exosome generation, several stimuli such as cellular stress, irradiation, hypoxia, or starvation have been shown to increase exosome production. Details regarding the mechanisms of exosomes biogenesis at a molecular level have been reviewed [56••]. Currently, starvation is the only method that has been used in exosome manufacturing [57••,58••].

Mechanisms of MP formation

MP formation requires cytoskeletal reorganization, especially as related to actin filaments. Inhibition of actin polymerization affected MP generation by different cell types. Treatment with cytochalasin D during platelet or neutrophil activation decreased MP formation [59,60]. However, latrunculin A led to a 2.4-fold increased MkMP generation [39]. ROCK-II (rho-associated coiled-coil-containing protein kinases 2) mediates thrombin-mediated erythrocytic-MP generation, with Caspase-2 as a ROCK-II activator [61]. Caspase-3 mediates ROCK-I cleavage and subsequently increased phosphorylation of myosin light chain and membrane blebbing [62]. In some cases, this is followed by flipping of phosphatidylserine (PS) from the inner to the outer leaflet of plasma membrane as mediated by the enzymes flippase, floppase, and scramblase [63]. Calcium appears to be involved in MP generation, as well. The calcium ionophore A23187 was shown to increase cytosolic Ca^{2+}, and to activate calpains involved in PMP formation [64].

Inducers of MP formation

MP formation can be stimulated by simple or complex chemicals, growth factors or other stimuli. For example, thrombin induces PMP [65] or endothelial-derived MP [61] generation. We have previously demonstrated shear-force-induced MkMP formation *in vitro*, apparently mediated by PS exposure and caspase-3 and caspase-9 activation [27,66]. The calcium ionophore A23187 has been used to increase Ca^{2+} influx and biogenesis of PMPs [67] and erythrocytic MPs [68]. TNF-α stimulated neutrophil-derived MP formation [69], giving rise to two distinct types of MPs from human aortic endothelial cells: one population is ROCK-dependent, miRNA-rich MPs, and the other population is caspase-dependent, miRNA-poor MPs [70]. Lastly, lipopolysaccharide was used to stimulate MP generation from monocytes or granulocytes [71].

Biomanufacturing of EVs: exosomes and MPs

EVs are currently pursued by several startup and larger companies for a broad range of applications, from minimally invasive diagnostic applications to therapeutic

interventions, including cell therapies and cargo delivery. To achieve large-scale EV production, it is necessary to develop EV manufacturing using Good Manufacturing Practices (GMP). Currently, several approaches have been reported for GMP-grade manufacturing of exosomes either from MSCs [58••,72] or from cardiac progenitor cells [57••]. The transition from bench-scale to large-scale exosome production for clinical use has been recently reviewed [73••]. These exosome manufacturing protocols are all based on surface-attached (anchorage-dependent) cells, which are generated in small or larger scale using technologies that have been developed since the 1980s to expand these cell types, and whereby exosomes are collected late in culture under some nutrient-starvation protocol. However, very little has been done in GMP-manufacturing of the larger MPs (MVs) from either a systems or mechanistic point of view.

In a systems-analysis examination that applies to all EVs, as shown in Figure 1, the first step is to identify which cell type to target. For clinical applications, one could target tumor cells aiming to enhance cell death, or target normal cells to impart desirable positive phenotypic responses, such as promoting cell proliferation or cell differentiation or to rescue cells from injury. Next, according to the biology of EV-to-target-cell interaction, one or more specific types of EVs will be selected for initial examination based on target specificity and biological outcomes in preclinical studies before narrowing the selection to one EV type for scaling up. If there are no options among naturally targeting EVs, one could engineer the surface characteristics of EVs to enhance the uptake process from the corresponding receptors presented by target cells. These EVs can be selected from easy-to-generate EVs such as MSC-EVs [74] or HEK-derived EVs. Once the EV source cells are selected, methods for EV biogenesis should be optimized. As discussed above, several mechanisms are known to and have been used to induce EV biogenesis. Among them, controlled biomechanical forces show a great potential for producing EVs. This can be executed quite readily for both suspension cultures [75,76] as well as for cultures of anchorage-dependent cells [77,78]. Scaling up the EV production process includes the following: (a) manufacturing EV-producing cells in a bioreactor of suitable scale; (b) applying an optimized protocol to induce EV formation; (c) optimizing of EV collection and purification process; (d) developing assays for and testing the biological of GMP-grade EVs; and (e) developing technologies for storage and freezing of EVs. Biomanufacturing will require detailed characterization of the cellular and functional EV properties to enable to identify and optimize key design and operational features of the culture system for a scalable and reproducible manufacturing process. Assays for detailed characterization of EVs based on physicochemical properties (size, density), surface-protein expression, and intraparticle content, and understanding how these properties relate to biological effectiveness will be necessary for EV manufacturing. These assays are to assess functional product 'quality', and thus to establish a 'process' versus 'function' relationship to address the question if and how does the manufacturing process affect product quality. For example, given that mixing and agitation in cell culture bioreactors affect expression of surface proteins and RNA content [79], it is clear that scaling up must be optimized to achieve the desirable EV content and surface protein expression. This issue has not yet been explored in the MP literature, but is widely recognized as important in the emerging cell-therapy industry. This is analogous to the issue to protein glycosylation of protein therapeutics that was brought to the forefront (and remains at the forefront) of manufacturing protein therapeutics, whereby the process profoundly affects protein glycosylation and thus therapeutic efficacy.

Future developments

Understanding the mechanisms of EV-to-target recognition and its specificity is crucial for developing more effective technologies for EV-based therapies or for using parts of the EVs or their parent cells to construct semi-synthetic delivery systems. Are there any reasons as to why some EV has exquisite target specificity while other do not? Can we determine if a cell's EVs will have some specific targets? Can we use modular engineering of one EV type to specifically target different cell types? This would enable to use one single biomanufacturing process with post-manufacturing EV engineering to develop the final product.

Similarly, better understanding of mechanistic aspects of EV uptake by target cells will enable better technologies to enhance the uptake process and deliver EV cargo to the desirable target-cell location. For example, if the nucleus is the target location, one would expect that endocytotic EV uptake might be undesirable due to the likely EV-cargo degradation through endosomal processing. Instead, understanding the mechanism of EV fusion to the plasma membrane (Figures 1 and 2) might enable means to enhance fusion and suppress endocytotic uptake. A related issue is that the dichotomy of target recognition versus EV uptake. We have shown that PMPs, which similar ontogeny as MkMPs can recognize and bind to HSPCs, but are not taken up by HSPCs [22••]. The mechanism that underlies this observation is unknown, but the phenomenon most likely is not unique, and can be exploited in several possible ways, including the development of competitive inhibition of undesirable EV uptake by a specific cell type.

A related issue is the mechanism that determines EV stability in circulation and biodistribution post infusion into the patient. The process by which transplanted cells home to the desirable organ has been investigated in

some cases, but remains, generally, not well understood. How much of that knowledge applies to EVs is unknown.

It is also important to develop synthetic-biology tools and vesicle technologies to enable combining components from different EVs or cell sources and synthetic membranes to create new synthetic vehicles. This would allow one to combine a broader spectrum of targeting mechanisms and cargo loading. Could one embed specific antibodies to the EV membrane for even more specific or enhanced target recognition? Could one load EVs with 'toxin' proteins to target cancer and other aberrant cells? Could one use EVs for targeted immunotherapy?

While some early efforts in exosome biomanufacturing have been reported, MP biomanufacturing remains largely unexplored. EV biomanufacturing would benefit from methods to quickly and accurately determine the loading efficiency of cargo (DNA, RNA, protein, or drugs) and the development of scalable, high-resolution purification methods, as well as technologies for product 'finishing', formulation, and storage. EV-product stability is also critical, as it would permit a wider spectrum of clinical applications, such as the case of the MkMPs, which can be stored frozen and could be possibly used as a possible substitute of platelet transfusions, to overcome the inability to store and transport platelets [22••].

Conflict of interest statement

The two authors are listed as inventors on a pending US/PCT patent application (publication US20170058262A1, Application No. 15/308,221, PCT No. PCT/US15/31388) on the MkMP-based technologies. The rights to the pending patent belong to the three inventors of the patent.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Tan X, Gong YZ, Wu P, Liao DF, Zheng XL: **Mesenchymal stem cell-derived microparticles: a promising therapeutic strategy**. *Int J Mol Sci* 2014, **15**:14348-14363.
2. Raposo G, Stoorvogel W: **Extracellular vesicles: exosomes, microvesicles, and friends**. *J Cell Biol* 2013, **200**:373-383.
3. Curtis AM, Edelberg J, Jonas R, Rogers WT, Moore JS, Syed W, Mohler ER 3rd: **Endothelial microparticles: sophisticated vesicles modulating vascular function**. *Vasc Med* 2013, **18**:204-214.
4. Gyorgy B, Szabo TG, Pasztoi M, Pal Z, Misjak P, Aradi B, Laszlo V, Pallinger E, Pap E, Kittel A *et al.*: **Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles**. *Cell Mol Life Sci* 2011, **68**:2667-2688.
5. Schwechheimer C, Kuehn MJ: **Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions**. *Nat Rev Microbiol* 2015, **13**:605-619.
6. El-Andaloussi S, Mager I, Breakefield XO, Wood MJ: **Extracellular vesicles: biology and emerging therapeutic opportunities**. *Nat Rev Drug Discov* 2013, **12**:347-357.
7. van Dongen HM, Masoumi N, Witwer KW, Pegtel DM: **Extracellular vesicles exploit viral entry routes for cargo delivery**. *Microbiol Mol Biol Rev* 2016, **80**:369-386.
8. Fais S, O'Driscoll L, Borras FE, Buzas E, Camussi G, Cappello F, Carvalho J, da Silva AC, Del Portillo H, El Andaloussi S *et al.*: **Evidence-based clinical use of nanoscale extracellular vesicles in nanomedicine**. *ACS Nano* 2016, **10**:3886-3899.
9. McKelvey KJ, Powell KL, Ashton AW, Morris JM, McCracken SA: **Exosomes: mechanisms of uptake**. *J Circ Biomark* 2015, **4**:7.
10. Mulcahy LA, Pink RC, Carter DR: **Routes and mechanisms of extracellular vesicle uptake**. *J Extracell Vesicles* 2014, **3**:24641.
11. Ghosh A, Li W, Febbraio M, Espinola RG, McCrae KR, Cockrell E, Silverstein RL: **Platelet CD36 mediates interactions with endothelial cell-derived microparticles and contributes to thrombosis in mice**. *J Clin Invest* 2008, **118**:1934-1943.
12. Janowska-Wieczorek A, Majka M, Kijowski J, Baj-Krzyworzeka M, Reca R, Turner AR, Ratajczak J, Emerson SG, Kowalska MA, Ratajczak MZ: **Platelet-derived microparticles bind to hematopoietic stem/progenitor cells and enhance their engraftment**. *Blood* 2001, **98**:3143-3149.
13. Baj-Krzyworzeka M, Majka M, Pratico D, Ratajczak J, Vilaire G, Kijowski J, Reca R, Janowska-Wieczorek A, Ratajczak MZ: **Platelet-derived microparticles stimulate proliferation, survival, adhesion, and chemotaxis of hematopoietic cells**. *Exp Hematol* 2002, **30**:450-459.
14. Turturici G, Tinnirello R, Sconzo G, Geraci F: **Extracellular membrane vesicles as a mechanism of cell-to-cell communication: advantages and disadvantages**. *Am J Physiol Cell Physiol* 2014, **306**:C621-C633.
15. Faille D, El-Assaad F, Mitchell AJ, Alessi MC, Chimini G, Fusai T, Grau GE, Combes V: **Endocytosis and intracellular processing of platelet microparticles by brain endothelial cells**. *J Cell Mol Med* 2012, **16**:1731-1738.
16. Obregon C, Rothen-Rutishauser B, Gitahi SK, Gehr P, Nicod LP: **Exovesicles from human activated dendritic cells fuse with resting dendritic cells, allowing them to present alloantigens**. *Am J Pathol* 2006, **169**:2127-2136.
17. •• Horibe S, Tanahashi T, Kawauchi S, Murakami Y, Rikitake Y: **Mechanism of recipient cell-dependent differences in exosome uptake**. *BMC Cancer* 2018, **18**:47.
This study demonstrates that the exosome uptake mechanism varies with different recipient cells, but not donor cells, thus suggesting that the selectivity of exosome uptake depends on target cells.
18. •• Costa Verdera H, Gitz-Francois JJ, Schiffelers RM, Vader P: **Cellular uptake of extracellular vesicles is mediated by clathrin-independent endocytosis and macropinocytosis**. *J Controlled Release* 2017, **266**:100-108.
This study demonstrates that the uptake of EVs by HeLa cells via clathrin-independent endocytosis and macropinocytosis.
19. Feng D, Zhao WL, Ye YY, Bai XC, Liu RQ, Chang LF, Zhou Q, Sui SF: **Cellular internalization of exosomes occurs through phagocytosis**. *Traffic* 2010, **11**:675-687.
20. Tian T, Zhu YL, Zhou YY, Liang GF, Wang YY, Hu FH, Xiao ZD: **Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery**. *J Biol Chem* 2014, **289**:22258-22267.
21. del Conde I, Shrimpton CN, Thiagarajan P, Lopez JA: **Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation**. *Blood* 2005, **106**:1604-1611.
22. •• Jiang J, Kao CY, Papoutsakis ET: **How do megakaryocytic microparticles target and deliver cargo to alter the fate of hematopoietic stem cells?** *J Controlled Release* 2017, **247**:1-18.

Details are given in this study about the interactions (receptor-mediated recognition, membrane fusion, and endocytosis) between MkMPs and to hematopoietic stem/progenitor cells (HSPCs) using antibody blocking assays, scanning electron (SEM), transmission electron (TEM), and confocal microscopies. Probably this could be the first unambiguous demonstration of membrane fusion as an MP-uptake mechanism.

23. Prada I, Meldolesi J: **Binding and fusion of extracellular vesicles to the plasma membrane of their cell targets**. *Int J Mol Sci* 2016, **17**:1296.

24. Huang Y, Liu K, Li Q, Yao Y, Wang Y: **Exosomes function in tumor immune microenvironment**. *Adv Exp Med Biol* 2018, **1056**:109-122.

25. Kahlert C, Kalluri R: **Exosomes in tumor microenvironment influence cancer progression and metastasis**. *J Mol Med (Berl)* 2013, **91**:431-437.

26. •• Fu Q, Zhang Q, Lou Y, Yang J, Nie G, Chen Q, Chen Y, Zhang J, Wang J, Wei T *et al.*: **Primary tumor-derived exosomes facilitate metastasis by regulating adhesion of circulating tumor cells via SMAD3 in liver cancer**. *Oncogene* 2018, **37**:6105-6118.
This study shows transfer of SMAD3 mRNA and protein between hepatocellular carcinoma cells via tumor-derived exosomes, thus mediating cancer metastasis.

27. Jiang J, Woulfe DS, Papoutsakis ET: **Shear enhances thrombopoiesis and formation of microparticles that induce megakaryocytic differentiation of stem cells**. *Blood* 2014, **124**:2094-2103.

28. •• Lee YS, Jung WY, Heo H, Park MG, Oh SH, Park BG, Kim S: **Exosome-mediated ultra-effective direct conversion of human fibroblasts into neural progenitor-like cells**. *ACS Nano* 2018, **12**:2531-2538.
This study demonstrates reprogramming of human fibroblasts into neural progenitor cells via fibroblast-derived exosomes by delivering reprogramming factors.

29. Deregibus MC, Cantaluppi V, Calogero R, Lo Iacono M, Tetta C, Biancone L, Bruno S, Bussolati B, Camussi G: **Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA**. *Blood* 2007, **110**:2440-2448.

30. McNeer NA, Schleifman EB, Cuthbert A, Brehm M, Jackson A, Cheng C, Anandalingam K, Kumar P, Shultz LD, Greiner DL *et al.*: **Systemic delivery of triplex-forming PNA and donor DNA by nanoparticles mediates site-specific genome editing of human hematopoietic cells in vivo**. *Gene Ther* 2013, **20**:658-669.

31. Bruno S, Grange C, Deregibus MC, Calogero RA, Saviozzi S, Collino F, Morando L, Busca A, Falda M, Bussolati B *et al.*: **Mesenchymal stem cell-derived microvesicles protect against acute tubular injury**. *J Am Soc Nephrol* 2009, **20**:1053-1067.

32. •• Keshtkar S, Azarpira N, Ghahremani MH: **Mesenchymal stem cell-derived extracellular vesicles: novel frontiers in regenerative medicine**. *Stem Cell Res Ther* 2018, **9**:63.
This work reviews recent studies on the therapeutic effects of MSC-EVs in kidney, liver, cardiovascular, and neurological diseases, and their potential role in regenerative medicine.

33. •• Di Trapani M, Bassi G, Midolo M, Gatti A, Kamga PT, Cassaro A, Carusone R, Adamo A, Krampera M: **Differential and transferable modulatory effects of mesenchymal stromal cell-derived extracellular vesicles on T, B and NK cell functions**. *Sci Rep* 2016, **6**:24120.
This study demonstrates the target specificity of MSC-EVs to monocytes among all other lymphocytes.

34. Bruno S, Grange C, Collino F, Deregibus MC, Cantaluppi V, Biancone L, Tetta C, Camussi G: **Microvesicles derived from mesenchymal stem cells enhance survival in a lethal model of acute kidney injury**. *PLoS One* 2012, **7**:e33115.

35. Dong L, Pu Y, Zhang L, Qi Q, Xu L, Li W, Wei C, Wang X, Zhou S, Zhu J *et al.*: **Human umbilical cord mesenchymal stem cell-derived extracellular vesicles promote lung adenocarcinoma growth by transferring miR-410**. *Cell Death Dis* 2018, **9**:218.

36. • Wu X, Liu Z, Hu L, Gu W, Zhu L: **Exosomes derived from endothelial progenitor cells ameliorate acute lung injury by transferring miR-126**. *Exp Cell Res* 2018, **370**:13-23.
This study demonstrates delivery of miR-126 to endothelial cells, *in vitro* and *in vivo*, through endothelial progenitor cell-derived exosomes (EPC-Exos).

37. Zhou Y, Li P, Goodwin AJ, Cook JA, Halushka PV, Chang E, Fan H: **Exosomes from endothelial progenitor cells improve the outcome of a murine model of sepsis**. *Mol Ther* 2018, **26**:1375-1384.

38. Cantaluppi V, Gatti S, Medica D, Figliolini F, Bruno S, Deregibus MC, Sordi A, Biancone L, Tetta C, Camussi G: **Microvesicles derived from endothelial progenitor cells protect the kidney from ischemia-reperfusion injury by microRNA-dependent reprogramming of resident renal cells**. *Kidney Int* 2012, **82**:412-427.

39. Flaumenhaft R, Dilks JR, Richardson J, Alden E, Patel-Hett SR, Battinelli E, Klement GL, Sola-Visner M, Italiano JE Jr: **Megakaryocyte-derived microparticles: direct visualization and distinction from platelet-derived microparticles**. *Blood* 2009, **113**:1112-1121.

40. Tao SC, Guo SC, Zhang CQ: **Platelet-derived extracellular vesicles: an emerging therapeutic approach**. *Int J Biol Sci* 2017, **13**:828-834.

41. •• Kuravi SJ, Harrison P, Rainger GE, Nash GB: **Ability of platelet-derived extracellular vesicles to promote neutrophil-endothelial cell interactions**. *Inflammation* 2018:1-16.
This study shows differential targeting of platelet EVs to neutrophils or endothelial cells through different surface proteins.

42. Ohno S, Takanashi M, Sudo K, Ueda S, Ishikawa A, Matsuyama N, Fujita K, Mizutani T, Ohgi T, Ochiya T *et al.*: **Systemically injected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells**. *Mol Ther* 2013, **21**:185-191.

43. Akao Y, Iio A, Itoh T, Noguchi S, Itoh Y, Ohtsuki Y, Naoe T: **Microvesicle-mediated RNA molecule delivery system using monocytes/macrophages**. *Mol Ther* 2011, **19**:395-399.

44. Zhang Y, Li L, Yu J, Zhu D, Zhang Y, Li X, Gu H, Zhang CY, Zen K: **Microvesicle-mediated delivery of transforming growth factor beta1 siRNA for the suppression of tumor growth in mice**. *Biomaterials* 2014, **35**:4390-4400.

45. Mizrak A, Bolukbasi MF, Ozdener GB, Brenner GJ, Madlener S, Erkan EP, Strobel T, Breakefield XO, Saydam O: **Genetically engineered microvesicles carrying suicide mRNA/protein inhibit schwannoma tumor growth**. *Mol Ther* 2013, **21**:101-108.

46. Fuhrmann G, Serio A, Mazo M, Nair R, Stevens MM: **Active loading into extracellular vesicles significantly improves the cellular uptake and photodynamic effect of porphyrins**. *J Controlled Release* 2015, **205**:35-44.

47. Tang K, Zhang Y, Zhang H, Xu P, Liu J, Ma J, Lv M, Li D, Katirai F, Shen GX *et al.*: **Delivery of chemotherapeutic drugs in tumour cell-derived microparticles**. *Nat Commun* 2012, **3**:1282.

48. Wahlgren J, De LKT, Brisslert M, Vaziri Sani F, Telemo E, Sunnerhagen P, Valadi H: **Plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes**. *Nucleic Acids Res* 2012, **40**:e130.

49. Cooper JM, Wiklander PB, Nordin JZ, Al-Shawi R, Wood MJ, Vithlani M, Schapira AH, Simons JP, El-Andaloussi S, Alvarez-Erviti L: **Systemic exosomal siRNA delivery reduced alpha-synuclein aggregates in brains of transgenic mice**. *Mov Disord* 2014, **29**:1476-1485.

50. Lamichhane TN, Raiker RS, Jay SM: **Exogenous DNA loading into extracellular vesicles via electroporation is size-dependent and enables limited gene delivery**. *Mol Pharm* 2015, **12**:3650-3657.

51. Momen-Heravi F, Bala S, Bukong T, Szabo G: **Exosome-mediated delivery of functionally active miRNA-155 inhibitor to macrophages**. *Nanomedicine* 2014, **10**:1517-1527.

52. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ: **Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes**. *Nat Biotechnol* 2011, **29**:341-345.

53. •• Kao CY, Papoutsakis ET: **Engineering human megakaryocytic microparticles for targeted delivery of nucleic acids to**

hematopoietic stem and progenitor cells. *Sci Adv* 2018, **4** eaau6762.
This study demonstrates first functional plasmid DNA delivery through MPs to hematopoietic stem/progenitor cells (HSPCs) and their nucleus.

54. Kanada M, Bachmann MH, Hardy JW, Frimannson DO, Bronsart L, Wang A, Sylvester MD, Schmidt TL, Kaspar RL, Butte MJ *et al.*: **Differential fates of biomolecules delivered to target cells via extracellular vesicles**. *Proc Natl Acad Sci U S A* 2015, **112**: E1433-E1442.

55. Tian Y, Li S, Song J, Ji T, Zhu M, Anderson GJ, Wei J, Nie G: **A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy**. *Biomaterials* 2014, **35**:2383-2390.

56. •• Hessvik NP, Llorente A: **Current knowledge on exosome biogenesis and release**. *Cell Mol Life Sci* 2018, **75**:193-208.
This work reviews studies on exosome biogenesis, from formation to release, including mechanistic and methodological aspects.

57. •• Andriolo G, Provasi E, Lo Cicero V, Brambilla A, Soncin S, Torre T, Milano G, Biemmi V, Vassalli G, Turchetto L *et al.*: **Exosomes from human cardiac progenitor cells for therapeutic applications: development of a GMP-grade manufacturing method**. *Front Physiol* 2018, **9**:1169.
It is the first example of biomanufacturing protocol for exosomes derived from human cardiac progenitor cells.

58. •• Mendt M, Kamerkar S, Sugimoto H, McAndrews KM, Wu CC, Gagea M, Yang S, Blanko EVR, Peng Q, Ma X *et al.*: **Generation and testing of clinical-grade exosomes for pancreatic cancer**. *JCI Insight* 2018, **3**:e99263.
This work describes the development of biomanufacturing and characterization protocol for exosomes derived from mesenchymal stem cells (MSC-EVs).

59. Thom SR, Bhopale VM, Yang M: **Neutrophils generate microparticles during exposure to inert gases due to cytoskeletal oxidative stress**. *J Biol Chem* 2014, **289**:18831-18845.

60. Yano Y, Kambayashi J, Shiba E, Sakon M, Oiki E, Fukuda K, Kawasaki T, Mori T: **The role of protein phosphorylation and cytoskeletal reorganization in microparticle formation from the platelet plasma membrane**. *Biochem J* 1994, **299**:303-308.

61. Sapet C, Simoncini S, Loriod B, Puthier D, Sampol J, Nguyen C, Dignat-George F, Anfosso F: **Thrombin-induced endothelial microparticle generation: identification of a novel pathway involving ROCK-II activation by caspase-2**. *Blood* 2006, **108**:1868-1876.

62. Sebbagh M, Renvoize C, Hamelin J, Riche N, Bertoglio J, Breard J: **Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing**. *Nat Cell Biol* 2001, **3**:346-352.

63. Morel O, Jesel L, Freyssinet JM, Toti F: **Cellular mechanisms underlying the formation of circulating microparticles**. *Arterioscler Thromb Vasc Biol* 2011, **31**:15-26.

64. Pasquet JM, Dachary-Prigent J, Nurden AT: **Calcium influx is a determining factor of calpain activation and microparticle formation in platelets**. *Eur J Biochem* 1996, **239**:647-654.

65. Xiao H, Jepkorir CJ, Harvey K, Remick DG: **Thrombin-induced platelet microparticles improved the aggregability of cryopreserved platelets**. *Cryobiology* 2002, **44**:179-188.

66. Luff SA, Kao CY, Papoutsakis ET: **Role of p53 and transcription-independent p53-induced apoptosis in shear-stimulated megakaryocytic maturation, particle generation, and platelet biogenesis**. *PLoS One* 2018, **13**:e0203991.

67. Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL: **Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin**. *Blood* 2000, **95**:1694-1702.

68. Salzer U, Hinterdorfer P, Hunger U, Borken C, Prohaska R: **Ca^{++}-dependent vesicle release from erythrocytes involves stomatin-specific lipid rafts, synexin (annexin VII), and sorcin**. *Blood* 2002, **99**:2569-2577.

69. Johnson BL 3rd, Goetzman HS, Prakash PS, Caldwell CC: **Mechanisms underlying mouse TNF-alpha stimulated neutrophil derived microparticle generation**. *Biochem Biophys Res Commun* 2013, **437**:591-596.

70. Alexy T, Rooney K, Weber M, Gray WD, Searles CD: **TNF-alpha alters the release and transfer of microparticle-encapsulated miRNAs from endothelial cells**. *Physiol Genomics* 2014, **46**:833-840.

71. Khaspekova SG, Antonova OA, Shustova ON, Yakushkin VV, Golubeva NV, Titaeva EV, Dobrovolsky AB, Mazurov AV: **Activity of tissue factor in microparticles produced in vitro by endothelial cells, monocytes, granulocytes, and platelets**. *Biochemistry (Mosc)* 2016, **81**:114-121.

72. Pachler K, Lener T, Streif D, Dunai ZA, Desgeorges A, Feichtner M, Oller M, Schallmoser K, Rohde E, Gimona M: **A good manufacturing practice-grade standard protocol for exclusively human mesenchymal stromal cell-derived extracellular vesicles**. *Cytotherapy* 2017, **19**:458-472.

73. •• Colao IL, Corteling R, Bracewell D, Wall I: **Manufacturing exosomes: a promising therapeutic platform**. *Trends Mol Med* 2018, **24**:242-256.
This reviews the possible methods for exosome biomanufacturing.

74. Lopatina T, Bruno S, Tetta C, Kalinina N, Porta M, Camussi G: **Platelet-derived growth factor regulates the secretion of extracellular vesicles by adipose mesenchymal stem cells and enhances their angiogenic potential**. *Cell Commun Signal* 2014, **12**:26.

75. Collins PC, Miller WM, Papoutsakis ET: **Stirred culture of peripheral and cord blood hematopoietic cells offers advantages over traditional static systems for clinically relevant applications**. *Biotechnol Bioeng* 1998, **59**:534-543.

76. Kunas KT, Papoutsakis ET: **Damage mechanisms of suspended animal cells in agitated bioreactors with and without bubble entrainment**. *Biotechnol Bioeng* 2009, **102**:980-987.

77. Cherry R, Papoutsakis E: **Hydrodynamic effects on cells in agitated tissue culture reactors**. *Bioprocess Eng* 1986, **1**:29-41.

78. Cherry RS, Papoutsakis ET: **Physical mechanisms of cell damage in microcarrier cell culture bioreactors**. *Biotechnol Bioeng* 1988, **32**:1001-1014.

79. McDowell CL, Papoutsakis ET: **Increased agitation intensity increases CD13 receptor surface content and mRNA levels, and alters the metabolism of HL60 cells cultured in stirred tank bioreactors**. *Biotechnol Bioeng* 1998, **60**:239-250.

80. Straat M, van Hezel ME, Boing A, Tuip-De Boer A, Weber N, Nieuwland R, van Bruggen R, Juffermans NP: **Monocyte-mediated activation of endothelial cells occurs only after binding to extracellular vesicles from red blood cell products, a process mediated by beta-integrin**. *Transfusion* 2016, **56**:3012-3020.