Extracellular vesicles (EVs) are biocompatible, nano-sized secreted vesicles containing many types of biomolecules, including proteins, RNAs, DNAs, lipids, and metabolites. Their low immunogenicity and ability to functionally modify recipient cells by transferring diverse bioactive constituents make them an excellent candidate for a next-generation drug delivery system. Here, the recent advances in EV biology and emerging strategies of EV bioengineering are summarized, and the prospects for clinical translation of bioengineered EVs and the challenges to be overcome are discussed.

1. Introduction

Extracellular vesicles (EVs) are heterogeneous nano-sized membrane vesicles that are released from various cell types. Classically, there are three major subtypes of EVs defined on the basis of their biological pathways, i.e., exosomes, microvesicles, and apoptotic bodies. Exosomes are vesicles formed by inward invagination of the endosomal membrane and fusion of the multivesicular body (MVB) with the plasma membrane. Microvesicles are formed by direct outward budding from the plasma membrane of healthy cells. Apoptotic bodies are formed during apoptosis by outward blebbing of the plasma membrane of cells. With the advance of EV studies, diverse EV subtypes have been proposed, using terms such as ectosomes, microparticles, oncosomes, etc., in addition to the three major types mentioned above. The different terminologies based on function, biogenesis, size, or cell of origin, as well as the variety of isolation methods and contexts, have led to various misconceptions and even contradictory definitions in the literature. In this context, the International Society for Extracellular Vesicles (ISEV) has proposed the term “extracellular vesicle” as generic nomenclature for cell-released vesicles, and has also recommended minimal requirements for EV studies. In addition, the EV-TRACK platform has been launched to facilitate standardization of EV research through more systematic reporting of EV biology and methodology.

In this review, we will use EVs as a generic term for the entire population of vesicles secreted from cells, since most research described in the literature has utilized heterogeneous populations of EVs, and often failed to characterize the isolated EVs in detail. EVs were originally discovered by Clargaff and West in 1946 as procoagulant platelet-derived particles in normal plasma. Since then, the biology and functions of EVs have been widely investigated (for example, see refs. [13,14] reviewed in refs. [1,15]). In 1983, EVs derived from fusion of MVBs with the plasma membrane of reticulocytes were observed. Subsequently, EVs were implicated in immune regulation and transfer of genetic materials, highlighting their role in cell–cell communication. It has been established that EVs contain thousands of different biomolecules, including proteins, RNAs, DNAs, lipids, and metabolites, and are highly heterogeneous in terms of their size, content, functional impacts on recipient cells, and cell of origin. Here, we focus mainly on the most extensively studied EVs, typically designated as exosomes and microvesicles (ectosomes), which play key roles in intercellular communication by delivering signals to recipient cells. These EVs hold great promise for developing next-generation delivery vehicles for therapeutic agents.

2. EV Biogenesis and Secretion

EVs are formed through multiple mechanisms (Figure 1). Classically, exosome biogenesis is initiated from the endosomal pathway, i.e., the formation of early endosomes via invagination of the plasma membrane. In some cases, vesicles derived from the budding of the trans-Golgi network (TGN) can fuse with early endosomes. During the following maturation process, early endosomes fuse to form late endosomes, leading to invagination of the endosomal membrane into the lumen and form intraluminal vesicles (ILVs). This in turn leads to the formation of multivesicular endosomes or MVBs with a characteristic multivesicular appearance. MVBs ultimately fuse with the plasma membrane and release the ILVs into the extracellular milieu in the form of exosomes. Alternatively, MVBs
Figure 1. Biogenesis and secretion of EVs. Two mechanisms of EV biogenesis are illustrated. The process of releasing exosomes into the extracellular milieu contains three distinct steps: exosome biogenesis, intracellular trafficking of MVBs, and fusion of MVBs with the plasma membrane. Early endosomes are formed by the inward budding of the plasma membrane (Step 1a1), or in some cases from the trans-Golgi network (TGN) (Step 1a2). Early endosomes mature into late endosomes (Step 1b) and finally generate MVBs, in which process ILVs are formed by inward invagination of the endosomal limiting membrane (Step 2). The fate of MVBs can be fusion with the plasma membrane (Step 3a1), which results in the release of exosomes (Step 3a2). Alternatively, MVBs can fuse with lysosomes/autophagosomes for degradation (Step 3b1, 2). Several molecules are involved in the biogenesis (e.g., RABs, ESCRTs, syndecan, ceramide, tetraspanins, etc.), trafficking (e.g., RABs, actin, etc.), and fusion of MVBs with the plasma membrane (e.g., SNAREs). Microvesicles arise from the direct outward budding and fission of the plasma membrane. Several molecules are involved in the biogenesis and release of microvesicles (small GTPases, ESCRTs, ARRD1C1, etc.). Abbreviations: EV, extracellular vesicle; ESCRT, endosome sorting complex required for transport; MVB, multivesicular body; ILV, intraluminal vesicle; RAB, RAS-related protein; ALIX, ALG-2 interacting protein X; nSMase2, neutral sphingomyelinase 2; Ral-1, RAL (Ras-related GTPase) homolog; SNARE, soluble NSF attachment protein receptor; VAMP7, vesicle-associated membrane protein 7; SNAP23, synaptosomal-associated protein 23; Syx1A, syntaxin 1A; ARF, ADP ribosylation factor; RhoA, Ras homolog family member A; A-SMase, acid sphingomyelinase; ARRD1C1, arrestin domain containing protein 1.*; homologs in C. elegans.

can fuse with lysosomes/autophagosomes to be degraded.[5,25] Briefly, the generation of exosomes consists of three steps: biogenesis, transport and release. Microvesicles (MVs) are derived from the plasma membrane by direct outward budding and fission, which is reminiscent of a reverse version of endocytosis.[5,27]

2.1. MVB Biogenesis

The endosome sorting complexes required for transport (ESCRT) machinery plays an important role in the formation of MVBs and ILVs (Figure 1). The ESCRTs comprise four distinct complexes (ESCRT-0, -I, -II, and -III) and the accessory Vps4 complex, with each of them consisting of several subunits (Box 1) (reviewed in refs. [29–31]).

In the canonical ESCRT-dependent pathway of ILVs (Figure 2A), the ESCRT complexes are recruited to the endosomal membrane in a stepwise manner. First, phosphatidylinositol-3-phosphate (PtdIns3P), an abundant phosphoinositide in endosomal membranes,[32,33] recruits the ESCRT-0 complex to early endosomes via its HRS (Vps27 in yeast) subunit.[34] Subsequently, HRS recruits clathrin,[35] which induces clustering of HRS to restricted microdomains.[36] HRS also binds to ubiquitin, which is essential for efficient sorting of ubiquitinated proteins into clathrin-coated microdomains.[37,38] Then, ESCRT-0
The endosomal sorting complexes required for transport (ESCRT) machinery was originally identified in budding yeast through genetic and biochemical characterization of vacuolar protein sorting (vps) mutants. ESCRT complexes have been extensively studied in yeast and human, and comprise ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III subcomplexes and ATPase complex, as well as several accessory proteins (see Table). Among them, ESCRT-0, ESCRT-I, ESCRT-II and ATPase complex are stable complexes existing before recruitment, while ESCRT-III subunits are dynamically assembled after recruitment. The ESCRT machinery plays a vital role in a series of membrane remodeling events, including multivesicular body (MVB) biogenesis, cytokinetic abscission and viral budding. The table lists the main components involved in the ESCRT pathway.

| Complex | Homo sapiens Component | Saccharomyces cerevisiae | Membrane Interaction | Intracomplex Interaction | Intercomplex Interaction |
|---------|------------------------|--------------------------|----------------------|--------------------------|--------------------------|
| ESCRT-0 | STAM1, 2               | Hse1                     | HRS                  | HD-PTP                   | TSG101, clathrin          |
|         | HRS                    |                          |                      |                          |                          |
| ESCRT-I | TSG101 (VPS23)         | Vps23                    | VPS28, VPS37, MVB12  | HRS, ALIX, HD-PTP        | EAP45, CHMP6              |
|         | VPS28                  | Vps28                    | TSG101               |                          |                          |
|         | VPS37A, B, C, D        | Vps37                    | TSG101, MVB12        |                          |                          |
|         | MVB12A, B              | Mvb12                    | TSG101, VPS37        |                          |                          |
|         | UBA1                   |                          |                      |                          |                          |
| ESCRT-II | EAP30 (VPS22)         | Vps22                    | EAP20, EAP45         |                          |                          |
|         | EAP20 (VPS25)          | Vps25                    | EAP30, EAP45         | CHMP6                    |                          |
|         | EAP45 (VPS36)          | Vps36                    | EAP30, EAP45         | CHMP6                    |                          |
| ESCRT-III | CHMP2A, B (VPS2A, B)   | Vps2                     | CHMP1, 3             | VPS4, LIP5               |                          |
|         | CHMP3 (VPS24)          | Vps24                    | CHMP2, 4             | VPS4                     |                          |
|         | CHMP4A, C, B (SNF7A, B, C) | Vps32 (Snf7)         | CHMP3, 6, 7          | VPS4, ALIX               |                          |
|         | CHMP6 (VPS20)          | Vps20                    | Myristoylation       | CHMP4                    | VPS4, EAP20, VPS28, HD-PTP |
|         | CHMP1A, B (DID2)       | Vps46 (Did2)             | CHMP2                | VPS4, EAP20, VPS28, HD-PTP |
|         | CHMP5 (VPS60)          | Vps60                    |                      | CHMP4                    |                          |
|         | CHMP7                  |                          |                      |                          |                          |
| ATPase  | VPS4A, B (SKD1)        | Vps4                     | LIP                  | CHMP1-6                  |                          |
|         | LIP5                   | Vta1                     | VPS4                 | CHMP1, 2, 5              |                          |
| Accessory | ALIX                  | Bro1                     | LBPA                 | TSG101, CHMP4             |
|         | HD-PTP                |                          |                      | UBA1, TSG101, CHMP4, STAM |

Abbreviations: STAM, signal transducing adaptor molecule; Vps, vacuolar protein sorting; HRS, hepatocyte growth factor-regulated tyrosine kinase substrate; Hse1, heat shock element 1; TSG101, tumor susceptibility gene 101 protein; MVB12, multivesicular body subunit 12; UBA1, ubiquitin associated protein 1; CHMP, charged multivesicular body protein; SKD1, suppressor of K+ transport defect 1; LIP5, LYST-interacting protein 5; Vta1, vesicle trafficking 1; ALIX, ALG-2 interacting protein X; HD-PTP, His domain protein tyrosine phosphatase; PtdIns3P, phosphatidylinositol 3-phosphate; LBPA, lysobisphosphatidic acid. a) MVB12A, B and UBA1 are mutually exclusive.

recruits ESCRT-I to endosomal membranes via direct interaction of HRS with the TSG101 (Vps23 in yeast) subunit of ESCRT-I. Indeed, TSG101 also contains a ubiquitin-binding domain. In yeast, Vps23 (TSG101 in human) can cooperate with Vps27 (HRS in human) to increase the sorting efficiency of ubiquitinated cargos. Besides interacting with ESCRT-0, ESCRT-I also interacts with the ESCRT-II complex. In yeast, this link is achieved by the Vps28 and Vps36 subunits of ESCRT-I and ESCRT-II, respectively. The function of ESCRT-I and ESCRT-II is thought to be responsible for endosomal membrane invagination. ESCRT-III assembly is initiated by ESCRT-II recruitment, which is mediated by the direct binding of Vps20 (CHMP6 in human) to Vps25 (EAP20 in human). ESCRT-III recruitment is required for ILV scission into the MVB lumen. After scission, ESCRT-III is disassociated from the membrane and recycled for additional rounds of budding. This dissociation process requires interaction with the AAA-ATPase Vps4. Vps24 might also cooperatively drive membrane scission.

There are other parallel ways to recruit ESCRTs for ILV biogenesis and cargo sorting, and these are termed non-canonical ESCRT-dependent pathways (Figure 1). Two non-canonical pathways have been identified in yeast, and four in mammals. In yeast, 1) Bro1 functions as a ubiquitin receptor and works in the ILV biogenesis pathway in parallel with ESCRT-0. 2) Bro1
Figure 2. MVB biogenesis machineries. Multiple molecular mechanisms of ILV generation in MVB have been revealed. A) In the canonical ESCRT-dependent pathway, ubiquitinated proteins in the endosomal membrane are recognized by ESCRT-0, which is recruited to the endosomal membrane by PtdIns3P binding and subsequently clustered into microdomains via clathrin binding. Then ESCRT-0 recruits ESCRT-I, and ESCRT-I recruits ESCRT-II. ESCRT-I and ESCRT-II coordinately induce the budding of the endosomal membrane and confine cargos within the buds. ESCRT-III components are dynamically recruited for membrane scission of the ILV necks and disassembled after ILV scission via VPS4. In a non-canonical ESCRT-dependent pathway, HD-PTP binds to ESCRT-0 and coordinately recruits ESCRT-I and ESCRT-III, bypassing the need for ESCRT-II. B) In the syndecan-syntenin-ALIX pathway, membrane budding and cargo clustering can occur independently of ubiquitin and ESCRT-0, but ESCRT-III and VPS4 are required for the scission step. C) Ceramide, generated from sphingomyelin by nSMase2, plays a key role in the ESCRT-independent pathway of ILV biogenesis. Ceramide can form lipid raft microdomains, which might trigger the conversion of ILVs into MVBs. D) CD63 plays a vital role in the ESCRT-independent pathway of ILV biogenesis. CD63 can form tetraspanin-enriched microdomains, which might trigger the conversion of ILVs into MVBs. Abbreviations: MVB, multivesicular body; ILV, intraluminal vesicle; ESCRT, endosome sorting complex required for transport; PtdIns3P, phosphatidylinositol 3-phosphate; STAM, signal transducing adaptor molecule; HRS, hepatocyte growth factor-regulated tyrosine kinase substrate; TSG101, tumor susceptibility gene 101 protein; VPS, vacuolar protein sorting; MVBl2, multivesicular body subunit 12; CHMP, charged multivesicular body protein; HD-PTP, His domain protein tyrosine phosphatase; ALIX, ALC-2 interacting protein X; nSMase2, neutral sphingomyelinase 2.

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and increased upon stimulation with C6 ceramide.\cite{83} Another ESCRT-independent pathway might be initiated by tetraspanins without the requirement of ubiquitination, ESCRT or ceramide \cite{86,87}\; (Figure 2D). In this mechanism, the tetraspanin CD63 is required for ILV formation and subsequent EV release,\cite{86,88}\; whereas depletion of ESCRT or ceramide does not impair EV secretion or cargo sorting.\cite{87} More recently, Wei et al. screened a library of constitutively active forms of Rab GTPases, and found that Rab31 controlled an ESCRT- and tetraspanin-independent, but ceramide-dependent ILV formation pathway.\cite{89,90}

As mentioned above, MVBs can fuse either with lysosomes for degradation of their contents or with plasma membrane for EV release\cite{5,25}\; (Figure 1). The mechanisms that determine the fate of MVBs are still largely unknown, but ISGylation of Tsg101 \cite{91}\; and tetraspanin 6 \cite{92}\; have been shown to negatively regulate the release of EVs. However, the reports on the effects of tetraspanin 6 on EV release are discrepant,\cite{92,93}\; possibly due to the differences of cell types and contexts. Similarly, there is a balance between EV secretion and macroautophagy; in the latter pathway, the autophagosomes subsequently fuse with lysosomes, resulting in degradation of their contents.\cite{5,25}\; (Figure 1). For example, inhibiting the fusion of MVBs with autophagosomes promotes the secretion of EVs.\cite{94,95}\; Moreover, it has recently been found that expression of several autophagy-related genes (Atg), including ATG3, ATG5, ATG7, ATG12, and LC3 (short for MAP1LC3B, microtubule-associated 1A/1B light chain 3B protein), promotes the biogenesis of MVBs and subsequent EV release independently of canonical macroautophagy.\cite{96–98}

### 2.2. MVB Transport

The transport of MVBs to the plasma membrane involves their interaction with the cytoskeleton, molecular motor and small GTPases,\cite{5,28,99}\; and shares similar mechanisms to those of other intracellular vesicles.\cite{100}\; The involvement of the cytoskeleton (microtubules and actin) in MVB transport is supported by the observation of oriented secretion of EVs in T cells and invasive cancer cells.\cite{82,101}\; The unidirectional transfer of EVs from T cells to antigen-presenting cells implies MVB trafficking along the network of microtubules in immunological synapses.\cite{101,102}\; The molecular motors involved in this process have not been identified, though actin was shown to provide docking sites for the intracellular trafficking of MVBs in cancer cells.\cite{82}\; Moreover, knockdown or overexpression of the actin cytoskeletal regulatory protein cortactin increases or decreases EV secretion, respectively.\cite{103}\; RAB GTPases are molecular switches that regulate intracellular vesicle transport,\cite{104}\; including EV secretion (Figure 1). RAB11 was the first RAB GTPase shown to be involved in EV secretion.\cite{105}\; Overexpression of a dominant-negative RAB11 mutant decreases the release of transferrin receptor (TfR) and increased upon stimulation with C6 ceramide.\cite{83} Another ESCRT-independent pathway might be initiated by tetraspanins without the requirement of ubiquitination, ESCRT or ceramide \cite{86,87}\; (Figure 2D). In this mechanism, the tetraspanin CD63 is required for ILV formation and subsequent EV release,\cite{86,88}\; whereas depletion of ESCRT or ceramide does not impair EV secretion or cargo sorting.\cite{87} More recently, Wei et al. screened a library of constitutively active forms of Rab GTPases, and found that Rab31 controlled an ESCRT- and tetraspanin-independent, but ceramide-dependent ILV formation pathway.\cite{89,90}

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### 2.3. Fusion of MVBs with the Plasma Membrane

The final step of exosome secretion is the fusion of MVBs to the plasma membrane, driven by soluble NSF attachment protein receptor (SNARE) proteins \cite{117,118}\; (Figure 1). The role of SNAREs in membrane fusion, including exocytosis, which is reminiscent of exosome secretion, has been extensively studied.\cite{118–120}\; The SNARE protein VAMP7, which is involved in fusion of secretory lysosomes with the plasma membrane,\cite{121}\; was shown to regulate the release of EVs in K562 leukemia cells,\cite{122}\; but inhibition of VAMP7 in MDCK cells has no effect on the secretion of EVs.\cite{123}\; Subsequently, two other SNAREs, syntaxin 1A (Syx1A) \cite{115}\; and Ykt6,\cite{124,125}\; were shown to be required for the secretion of EVs containing Evi and Wnt, respectively, in the tested cell types. Recently, SNAP-23 was demonstrated to facilitate EV release in tumor cells under the control of phosphorylation mediated by pyruvate kinase type M2 (PKM2).\cite{126}\; In C. elegans, the SNARE protein Syx-5 is involved in the fusion of MVBs to the plasma membrane, and its absence causes accumulation of MVBs beneath the plasma membrane.\cite{112}

### 2.4. Biogenesis and Release of Microvesicles

The molecular mechanisms of biogenesis of microvesicles are less well characterized. Microvesicles have been referred to as ubiquitous vesicles that are formed by direct budding from the plasma membrane of healthy cells \cite{1,7}\; (Figure 1). One mechanism of their synthesis involves the ESCRT machinery. Knockdown of ESCRT proteins, including Alix, Tsg101, Vps22, Chmp1/3, and Vps4 reduced the secretion of Hedgehog...
Figure 3. Uptake and fate of EVs. EVs can trigger intracellular signaling of recipient cells via ligand-receptor interaction, such as antigen presentation, immune modulation and morphogen signaling. Alternatively, exogenous EVs can transfer their cargos into recipient cells by entering the cells. EVs can be internalized into recipient cells by different mechanisms, including membrane fusion and various endocytic pathways (e.g., receptor-mediated endocytosis, caveolin-mediated endocytosis, lipid raft-mediated endocytosis, phagocytosis and macropinocytosis). The fusion of EVs with the plasma membrane can release their contents into the cytoplasm of recipient cells, while the endocytosed EVs reach MVBs via the canonical endosomal pathway. These internalized EVs might be degraded after the fusion of MVBs with lysosomes or be secreted from recipient cells mixed with endogenous ILVs (not shown). Also, they might back-fuse with the limiting membrane of MVBs, leading to the release of the EV cargos into the cytoplasm, a process that is poorly understood. Abbreviations: EV, extracellular vesicle; MVB, multivesicular body; ILV, intraluminal vesicle; TCR, T-cell receptor; MHC, major histocompatibility complex; PD1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; Fz, Frizzled receptor.

3. EV Uptake and Cell–Cell Communication

Accumulating evidence has demonstrated that EVs act as an important mediator of intercellular communication, and this has generated increasing interest in the development of EV-based therapeutic agents. It is generally accepted that EVs exert their intercellular signaling function in two ways: 1) they can transmit information to recipient cells by direct contact via their surface ligands; 2) they can transfer proteins and nucleic acids, including RNAs and DNAs to target cells.

The first mechanism depends on ligand–receptor interaction, without delivery of the EV contents into the recipient cell, and has been well studied in connection with immunomodulation (Figure 3). For example, EVs derived from B lymphocytes and dendritic cells (DCs) harbor major histocompatibility complex (MHC), and can provoke T cell-mediated immune responses by activating cognate T cell receptors. Another example is cancer cell-derived EVs bearing programmed death ligand-1 (PD-L1), which can lead to cancer immune evasion via inhibition of T cell function by binding to programmed cell death protein-1 (PD1).

(Hh) via EVs. Another mechanism involves recruitment of the ESCRT subunits TSG101 and VPS4 to the plasma membrane by adaptor protein arrestin domain-containing protein 1 (ARRDC1), thereby promoting the generation of EVs (termed ARRDC1-mediated microvesicles; ARMMs). Apart from the ESCRT machinery, small GTPase proteins, including ARF1, ARF6 and RhoA, also enable EVs to bud off from the plasma membrane of cancer cells. Moreover, activation of acid sphingomyelinase (A-SMase), another member of the SMase family on the plasma membrane, can trigger the release of EVs from glial cells and red blood cells via generation of ceramide.
Wnt protein-bearing EVs can induce Wnt signaling activity in recipient cells.\textsuperscript{[124,129]} This property indicates that EVs could be promising candidates for developing immunomodulatory therapeutics and antitumor vaccines.

The second mechanism depends on cellular internalization or membrane fusion, leading to the entry of the EV contents into acceptor cells (Figure 3). This mechanism has been extensively investigated in various cell types and is well characterized. For instance, EVs can transfer microRNAs (miRNAs) into acceptor cells to downregulate expression of target genes\textsuperscript{[21,22,101,140,141]} and deliver messenger RNAs (mRNAs) to be functionally translated.\textsuperscript{[20–22,142]} Other functionally important genetic materials, including genomic DNAs (gDNAs),\textsuperscript{[143,144]} mitochondrial DNAs (mtDNAs),\textsuperscript{[145,146]} and long noncoding RNAs (lncRNAs)\textsuperscript{[143,147]} can also be delivered to recipient cells via EVs. EVs can also transfer protein cargos, such as oncoproteins from cancer cells to neighboring cells\textsuperscript{[22,108,140,150–153]} and deliver a retrograde signal (i.e., synaptotagmin 4) from presynaptic to postsynaptic cells.\textsuperscript{[151]} Nevertheless, although the cargo delivery capacity of EVs has been demonstrated and delivery systems based on EVs have already been developed,\textsuperscript{[152]} the mechanisms of EV uptake and cargo delivery into the cytosol of recipient cells are still poorly understood.

Given that EV–cell interactions and virus–cell interactions share topological similarities, the mechanisms of viral uptake provide useful paradigms for exploring the mechanisms of EV uptake.\textsuperscript{[155,158]} Currently, two models of EV uptake have been proposed and are widely accepted: direct membrane fusion and endocytosis.\textsuperscript{[154,155]} Indeed, EV uptake via direct fusion with the cell’s plasma membrane has been observed directly using fluorescent lipid dequenching.\textsuperscript{[154]} Parolini et al. used a lipid fluorescent probe, R18, to label EVs derived from melanoma cells, and demonstrated that a part of these labeled EVs directly fused with the plasma membrane of acceptor cells.\textsuperscript{[156]} Subsequently, Montecalvo et al. obtained similar results in bone-marrow-derived DCs using the dequenching method, and further showed that EV uptake could occur via fusion of EVs with the plasma membrane.\textsuperscript{[157]} Nevertheless, the fusion-based pathway is not the main uptake mechanism. Most experimental evidence indicates that EVs are mainly taken up into endosomes via endocytosis.\textsuperscript{[157–160]} Endocytosis is a generic term for cellular internalization, which can be generally subdivided into five categories: receptor-mediated endocytosis (also known as clathrin-mediated endocytosis), caveolin-mediated endocytosis, lipid raft-mediated endocytosis, phagocytosis, and macropinocytosis.\textsuperscript{[154,155,161]} All of these mechanisms have been observed in various cell types (reviewed in refs. [154,155,162]).

Here, we focus on receptor-mediated endocytosis, because this uptake pathway is a highly specific process that can occur only when EVs and acceptor cells share the right combination of ligand and receptor. This feature offers the possibility of controlling the tropism of engineered EVs. Though EVs naturally possess a broad tropism,\textsuperscript{[152,163]} there are certainly examples of cell-type-specific uptake of EVs,\textsuperscript{[164,165]} and introducing a targeting moiety can markedly improve tropism for target cells.\textsuperscript{[163]} For example, Morelli et al. found that both EV tetraspanins CD9 and CD81 and cellular integrin \(\alpha_v/\beta_3\) contribute to EV uptake by DCs.\textsuperscript{[158]} Further, integrin CD11a and its ligand CD54 (also known as intercellular adhesion molecule 1, ICAM-1) on both EVs and DCs play a role in EV uptake by DCs.\textsuperscript{[158]} Nazarenko et al. reported that Tspan8-CD49 complex-containing EVs could be selectively internalized by endothelial cells in a process mediated by the ligand CD54 expressed on acceptor cells.\textsuperscript{[167]} Integrins \(\alpha_v/\beta_4\) and \(\alpha_v/\beta_3\) on tumor-derived EVs\textsuperscript{[165]} and scavenger receptors on endothelial cells and patrolling macrophages \textsuperscript{[160]} also mediated the cell-specific uptake of EVs. Another example of a receptor-mediated EV uptake pathway involves heparan sulfate proteoglycans (HSPGs) expressed on recipient cells.\textsuperscript{[164]} In all the aforementioned studies, chemical or antibody intervention or genetic deficiency reduced the EV uptake by recipient cells.

Thus far, many studies have demonstrated that EVs can be taken up via a receptor-mediated endocytosis pathway, which can be manipulated at least to some extent.\textsuperscript{[24]} However, due to the heterogeneity of EVs and the existence of plural coexisting uptake routes, there is still a long way to go to improve the tropism of EVs in order to achieve targeted delivery. Moreover, compared with direct fusion, endocytosis is a more common entry route of EVs, so the endosome/lysosome is a likely destination for EV-delivered cargo content.\textsuperscript{[155]} Nevertheless, several studies have shown that endocytosed EVs can functionally influence acceptor cells,\textsuperscript{[159,160,168]} indicating that EV-contained cargos can escape from the degradative pathway. In these cases, release of the cargos of internalized EVs might be achieved through a back-fusion process, in which EVs fuse with the limiting membrane of the MVB (Figure 3). However, the mechanism of this putative back-fusion process remains largely to be elucidated, and it is unclear how recipient cells discriminate EV contents to be functionally delivered or degraded.

4. Cargo Sorting into EVs

The ESCRT machinery plays a key role not only in the biogenesis of ILVs, but also in protein sorting, particularly for ubiquitinated cargos \textsuperscript{[170]} (Figure 2A). ESCRT-0, -I, -II have ubiquitin-binding modules that interact directly with ubiquitinated proteins and are necessary for cargo sorting.\textsuperscript{[171]} Both ESCRT-0 subunits, HRS and STAM (Vps27 and Hse1 in yeast), contain ubiquitin-interacting motifs (UIMs), which initiate the sorting of ubiquitinated membrane proteins.\textsuperscript{[176,177]} After clustering ubiquitinated cargos to clathrin-coated microdomains, ESCRT-0 hands them over to ESCRT-I and -II. Among the ESCRT-I subunits, both TSG101 (Vps23 in yeast) and MVB12/UBAP1 contain a ubiquitin-binding domain that binds ubiquitinated cargos, and this seems to be crucial for cargo sorting.\textsuperscript{[175,176] A} Among the ESCRT-II subunits, Vps36 (EAP45 in human) can bind ubiquitin,\textsuperscript{[175,176]} as well as ESCRT-I and lipid.\textsuperscript{[177,178]}

In one non-canonical ESCRT-dependent pathway, yeast Bro1 (ALIX in humans) functions as ubiquitin receptor, which sorts ubiquitinated cargos into ILVs, bypassing the need for ESCRT-0.\textsuperscript{[62]} In another mechanism, Bro1 functions as a bridge between ESCRT-0 and ESCRT-III in parallel to ESCRT-I and -II, and is also required for ubiquitinated cargo sorting.\textsuperscript{[64]} In the syndecan-syntenin-ALIX pathway in mammalian cells, CD63 interacts with syntenin,\textsuperscript{[179]} and syndecan-syntenin regulates the sorting of the tetraspan CD63 and its EV release \textsuperscript{[65]} (Figure 2B). In contrast, the sorting of tetraspanins CD9 and CD81, as well as fortillin-1,
is not affected by syndecan-syntenin.\textsuperscript{[56-58]} ALIX (Bro1 in yeast) can directly bind various proteins through its YPX\textsubscript{3}L motifs, and this drives cargo sorting in a way that is independent of ubiquitination as well as ESCRT-0 and -I, but depends on ESCRT-II.\textsuperscript{[69,180]} Alternatively, ALIX can sort tetraspanins (e.g., CD9, CD63 and CD81) into EVs regulated by ubiquitination, but not canonical ubiquitinated cargos (e.g., EGFR), in a LBPA-ESCRT-III-dependent pathway, which does not require ESCRT-0, -I, or -II.\textsuperscript{[73]} Another Bro1 domain-containing protein, HD-PTP can bypass the requirement for ESCRT-II and function as a scaffold that consecutively recruits ESCRT-0, -I, and -III.\textsuperscript{[74-78]} In this process, HD-PTP is required for ubiquitinated cargo sorting into ILVs and for MVB morphogenesis,\textsuperscript{[79,80]} but is dispensable for ILV formation.\textsuperscript{[76]}

ESCRT-III has no known ubiquitin binding ability, which is consistent with its role in vesicle budding and scission, but not in cargo sorting (Figure 2A,B). The ESCRT-III subunit, Vps24 (CHMP3 in human) can bind specifically to PtdIns3P,\textsuperscript{[181]} which might serve to stabilize ESCRT-III on membranes.

In the ESCRT-independent pathway, cargo sorting to ILVs of MVBs does not require ubiquitination or ESCRT complexes.\textsuperscript{[182,183]} In the ceramide-dependent and ESCRT-independent EV biogenesis pathway, ceramide forms a lipid raft-like microdomain, which can sort PLP into ILVs (Figure 2C). The sorting of CD63 as well as CD81 and flotillin to ILVs may be under the control of sphingosine 1-phosphate (SIP) signaling; SIP is a metabolite of ceramide.\textsuperscript{[184]} Meanwhile, these tetraspanins can regulate sorting of various cargos to ILVs.\textsuperscript{[81,84]} In the tetraspanin-dependent and ESCRT-independent EV biogenesis pathway, the protein PMEL is sorted to ILVs by interacting with the tetraspanin CD63.\textsuperscript{[86,87]} (Figure 2D). This cargo-sorting capacity of tetraspanins is also supported by other studies, though the exact EV biogenesis pathways in which they were involved are not well defined.\textsuperscript{[93,185-189]} Since tetraspanins are ubiquitous transmembrane proteins in late endosomes,\textsuperscript{[190]} and they can sort both ubiquitinated and non-ubiquitinated cargos into ILVs,\textsuperscript{[93,185,187,188]} they are thought to participate in various EV biogenesis pathways.

Apart from sorting of transmembrane proteins into ILVs, some cytosolic proteins can also be selectively targeted to EVs (Figure 2). For instance, Hsc70 is co-sorted into EVs along with TIR.\textsuperscript{[191,192]} Moreover, Hsc70 can selectively sequester cytosolic proteins containing a KFERQ-motif into ILVs.\textsuperscript{[193]} Cytosolic proteins β-catenin \textsuperscript{[81]} and apolipoprotein E (ApoE)\textsuperscript{[194]} can also be co-sorted into EVs with other transmembrane proteins. In addition, some cytosolic proteins modified by phosphorylation\textsuperscript{[194]} and farnesylation\textsuperscript{[195]} are sorted into ILVs and released by EVs, though the mechanisms involved are unclear.

Besides these sorted EVs, other EVs also contain a variety of nucleic acids, including RNAs and DNAs.\textsuperscript{[196,197]} (Figure 4). Initially, miRNAs and mRNAs were identified in EVs.\textsuperscript{[198-202]} Subsequently, many other kinds of RNAs were identified in EVs,\textsuperscript{[197-201]} including transfer RNAs (tRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), Y-RNAs, vault RNAs (vRNAs), lncRNAs,\textsuperscript{[143,147]} piwi-interacting RNAs (piRNAs), mitochondrial RNAs (mtRNAs) and circular RNAs (circRNAs).\textsuperscript{[202]} DNAs were also identified in EVs, including single-stranded DNAs (ssDNAs),\textsuperscript{[141]} double-stranded DNAs (dsDNAs)\textsuperscript{[203,204]} and mitochondrial DNAs (mtDNAs).\textsuperscript{[146]}

During the process of EV biogenesis, various proteins, including RNA-binding proteins (RBPs),\textsuperscript{[199]} are selectively sequestered into EVs.\textsuperscript{[203]} The RBPs contain a sequence-specific RNA-binding domain (RBD) that sorts a set of RNAs into EVs.\textsuperscript{[147,206-209]} For example, hnRNPA2B1 recognizes the GGAG/CCCU motifs in the 3’ end of miRNAs\textsuperscript{[206]} and the 5’ end of lncRNA (i.e., lncARS).\textsuperscript{[147]} which cause specific miRNAs and lncARS to be concentrated into EVs. Similarly, SYNCRIPI (also known as hnRNPO) directly binds to specific miRNAs and sorts them into EVs by recognizing a GCCU motif.\textsuperscript{[207,208]} Also, there are RBPs that facilitate miRNA sorting into EVs, though their RBDs have not been identified.\textsuperscript{[207,209]} Apart from RBPs, some components of the RNA-induced silencing complex (RISC) (e.g., Ago2) are present in EVs.\textsuperscript{[141,194,210]} Overall, these proteins cause a set of miRNAs to congregate into EVs. Other mechanisms also exist, but it is not clear whether RBPs/RISC are involved.\textsuperscript{[211,212]}
5. EV Bioengineering

Bioengineering of EVs employs genetic methodology to design and produce EVs with novel functionalities and properties based on knowledge of EVs biogenesis, secretion and uptake pathways (Figure 5). Currently, EV engineering capabilities include cargo loading and target delivery using genetic and nongenetic methods both in vitro and in vivo (reviewed in refs. [24,213,214]). In this review, we focus on the bioengineering of EVs—using molecular engineering techniques to manipulate the cellular machinery for boosting production of EVs, sorting cargos into EVs in parent cells and target EVs to the desired recipient cells.

Since the finding of that EVs could horizontally transfer miRNAs and mRNAs between cells,[20–22] and participate in cell–cell communication,[6] EVs have attracted increasing attention. The natural origin and biological properties of EVs are particularly advantageous for application of EVs as a specific, effective and safe delivery system.[134]

The initial target of EV bioengineering was simply to load small RNAs into EVs, inspired by the finding that EVs can deliver RNAs.[20–22] However, because the mechanisms that direct RNA sorting into EVs remained unclear, loading of small RNAs into EVs was primarily performed through cell transfection approaches. Overexpression of desired miRNAs in cells allowed these miRNAs to be incorporated into EVs and subsequently released.[215] Indeed, Kosaka et al. showed that miRNAs overexpressed in HEK293 and COS-7 cells could be released via a ceramide-dependent pathway.[215] A similar transfection-based approach was used to load therapeutic small RNAs into EVs in other studies.[216,217] More recently, the molecular mechanisms behind EV RNA sorting have been largely uncovered.[147,206,207,209,210,212,218,219] which has greatly facilitated EV engineering for RNA loading. In particular, it was found that various RBPs in EVs could recognize and bind different RNAs that are enriched in EVs.[220] These enriched RNAs share a common seed sequence, hereafter termed the EXOmotif, for their cognate RBPs. Loading into EVs can be enhanced by incorporating the EXOmotif into an RNA that normally would not be exported via EVs. For example, hnrRNP2A2B1, which is the first protein identified as being involved in EV miRNA sorting, recognizes the GGAG EXOmotif.[206] Mutations in this EXOmotif reduce the EV accumulation of miR-601, while introducing this EXOmotif into miR-17 increases its loading into EVs.[206] Another RBP SYNCRIP recognizes the GGCU EXOmotif, and insertion of this motif into miRNAs efficiently enhances the accumulation of chimera-miRNAs into EVs.[207] More recently, Reshke et al. showed that silencing RNA (siRNA) could be efficiently packaged into EVs via a pre-miRNA backbone, which greatly reduced the therapeutic dose of siRNA.[221] In their study, the pre-miRNA backbone itself was able to facilitate siRNA loading into EVs independently of luminal argonaute (Ago), as well as other reported RBPs.[221]

Apart from small RNAs, proteins and mRNAs can also be loaded into EVs through the application of EV bioengineering (Figure 5). One approach uses optogenetic methodology to load proteins of interest into engineered EVs via reversible protein–protein interaction dimersizers controlled by blue light (termed “EXPLORs”). This allows cargo proteins to be efficiently incorporated into EVs and released into the EV intraluminal space by switching the blue light on and off, respectively.[222] In a more recent study, Sterzenbach et al. showed that the late-domain (L-domain)-containing proteins involved in EV biogenesis could be adopted to recruit soluble proteins into EVs.[223] In their study, the WW-tagged Cre protein was efficiently loaded into EVs through
Bioengineering has also been applied to ARMMs, a type of EV distinct from exosomes that is produced by direct plasma membrane budding.\(^{[128]}\) ARMMs can deliver active Notch receptors to recipient cells and induce non-canonical intercellular Notch signaling.\(^{[237]}\) Wang et al. recently used ARMMs to efficiently deliver the tumor suppressor protein p53 in vivo, utilizing a chimeric protein fused to ARRD1C. Also, p53 mRNA was successfully loaded into ARMMs by fusing the transactivator of transcription (Tat) peptide to ARRD1C and fusing its RNA binding-motif, trans-activating response (TAR) element, to p53 mRNA. In addition, WW-Cas9 fusion protein robustly enables CRISPR-Cas9/guide RNA complex to be sorted into ARMMs via interaction with the PPxY motifs of ARRD1C, and delivered to recipient cells.\(^{[236]}\)

6. Conclusions and Perspectives

The processes of EV biogenesis and release are currently largely understood, and provide a rational basis for manipulating the production of EVs. Current knowledge of cargo sorting machineries also allows various therapeutic cargos, including RNAs and proteins, to be efficiently loaded into engineered EVs in genetically modified parental cells. Although EV uptake mechanisms are not yet completely understood, it is already possible to target EVs to desired target cells or tissues through engineered ligands/receptors on their surface. In this review, we have summarized the current knowledge of EV biogenesis mechanisms and bioengineering methodologies. Beyond that, there is also a flourishing field of EV engineering using nongenetic strategies in vitro (see, for example, refs. [238,239] reviewed in ref. [240]). In addition, there is another approach using artificial nanoparticles to deliver various therapeutic cargos to desired target tissues (see, for example, refs. [241–246]). Since these nanoparticles share many similarities with EVs, dialogue among these fields should facilitate bioengineering of EVs for therapeutic applications.

Nevertheless, our current knowledge of EV biogenesis, release, uptake and cargo sorting is still incomplete, and some pressing issues remain to be elucidated. 1) Various ESCRT-dependent and -independent pathways have been uncovered (as discussed above, and also reviewed in refs. [5,28]), but their contributions to EV biogenesis vary markedly depending on the cell type and/or cellular state. Extensive further investigations are still needed. 2) Some insights into the balance between exosome secretion and lysosomal degradation have emerged (see, for example, refs. [65,91,92,185]), but the mechanisms that regulate this balance are still poorly understood. 3) The loading machineries for some small RNAs and several proteins that are sorted into EVs have also been identified (discussed above), but their full scope is still largely unknown. 4) Molecules involved in MVB trafficking have been partially uncovered in particular exosome release pathways (reviewed in refs. [5,247]), but because they do not necessarily need to be incorporated into exosomes, many of them are still unidentified. 5) Molecules on the EV surface certainly influence recognition and capture by acceptor cells (reviewed in refs. [6,154]), but our knowledge of the fates of EVs and their delivered cargos remains limited. Advances in these areas will require new experimental approaches and technologies. More recently, the composition of EVs has been extensively reassessed, and some
previous findings have been overturned.\cite{248,249} In future, it will be necessary to interrogate EV composition at the single-vesicle level and also to dissect EV biogenesis at the single-cell level in order to provide a basis for proper design and engineering of EVs for therapeutic applications.

Furthermore, to apply bioengineered EVs for clinical applications, it will be essential to produce engineered EVs in clinically authorized cell types, such as human mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and so on. Another challenge is to scale-up EV production. Therefore, detailed characterization of EV biogenesis pathways and cargo-sorting machineries in these cell types will be needed. Confirmation of the applicability of current bioengineering strategies to these cell types is also required, because different strategies might be required for different cell types. For instance, the EXOtic device can boost production of EVs, thus reducing the necessary scale of cell culture in principle, but it was established in HEK-293T cells, and works with reduced efficiency in hMSCs.\cite{215} It will be important to establish whether it can be used in other stem cells. Since EVs possess natural tropism for the liver and spleen,\cite{161,231,250} it will also be important to alter the tropism and to improve specific targeting. The tropism of EVs for target cells and tissues has already been changed and improved through engineering the targeting ligands/receptors on the EV surface.\cite{161} For further improvement, more specific ligands/receptors will be needed, or it may be possible to integrate plural ligands/receptors on the surface of the same EV.

In conclusion, EVs are derived from cells and consequently possess excellent biocompatibility, biostability and low immunogenicity, which are highly desirable characteristics for a new therapeutic delivery system. The increasing exploration of EV biology is providing various possible strategies to design and engineer EVs for therapeutic purposes. For example, we could hijack proteins involved in cargo sorting to package various cargos, including the above-mentioned small RNAs, mRNAs and proteins, or even DNAs in the future, into engineered EVs through genetic modifications. We could fuse proteins retained on EVs to different target ligands/receptors in order to target EVs to desired tissues. Also, we could boost the production of EVs by genetically targeting ligands/receptors in order to target EVs to desired tissues. Finally, we could boost the production of EVs by genetically targeting ligands/receptors in order to target EVs to desired tissues.

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Conflict of Interest

The authors declare no conflict of interest.

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