Therapeutic and diagnostic applications of extracellular vesicles

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Abstract

During the past two decades, extracellular vesicles (EVs) have been identified as important mediators of intercellular communication, enabling the functional transfer of bioactive molecules from one cell to another. Consequently, it is becoming increasingly clear that these vesicles are involved in many (patho)physiological processes, providing opportunities for therapeutic applications. Moreover, it is known that the molecular composition of EVs reflects the physiological status of the producing cell and tissue, rationalizing their exploitation as biomarkers in various diseases. In this review the composition, biogenesis and diversity of EVs is discussed in a therapeutic and diagnostic context. We describe emerging therapeutic applications, including the use of EVs as drug delivery vehicles and as cell-free vaccines, and reflect on future challenges for clinical translation. Finally, we discuss the use of EVs as a biomarker source and highlight recent studies and clinical successes.

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1. Introduction

1.1. A brief historical overview of EVs

In addition to single molecules (i.e. small molecules, peptides and proteins), macromolecular complexes (e.g. Argonaute2 (AGO2)-RNA complex) and lipoproteins, cells also release membrane-enclosed vesicles in the extracellular medium. The first reports on such extracellular vesicles (EVs) date back to the late 1960s when it was observed that platelet free plasma contains vesicular material that could be pelleted down by ultracentrifugation (UC). These vesicles were mainly composed of phospholipids and appeared to promote blood clothing [1] and cartilage calcification [2]. In the decades that followed, using electron microscopy imaging, vesicular structures could be visualized in calf serum [3] and the first observations were made on tumor cell-derived membrane vesicles [4]. Initially it was assumed that the observed vesicles were solely released by outward budding of the cell membrane. Several years later, Johnstone and colleagues reported on the formation of intraluminal vesicles (ILVs) in late endosomes by inward budding of the endosomal limiting membrane. Following fusion of these so-called multivesicular bodies (MVBs) with the cell's plasma membrane, the ILVs are released in the surrounding fluid [5, 6]. This discovery was made based on the observation that reticulocytes release their transferrin receptor, as part of the maturation into erythrocytes, associated to vesicles. As this mechanism was also observed in other species and appeared to be selective for certain membrane-associated proteins [7], these EV were initially presented as a conserved and regulated waste removal pathway [8]. A seminal paper by Raposo *et al.* in 1996, reporting on the immune-modulating activity of B cell-derived EVs, inspired many others to evaluate the biological implications of these vesicles [9]. Two years later, Zitvogel *et al.* used EVs derived from tumor peptide-pulsed dendritic cells (DCs) as a cell-free anti-cancer vaccine providing the first therapeutic application of EVs [10]. Among others, these reports introduced the notion that EVs cannot solely be considered as a waste disposal

mechanism but also as important mediators of intercellular communication. Owing to the work of many, it was becoming increasingly clear that EVs likely play a fundamental role in many (patho)physiological processes. Besides deciphering the biological function of EVs, their potential as biomarker source [11, 12] was recognized and the first clinical trials using EVs as an anti-cancer vaccine were initiated [13]. In addition, around a decade ago different groups identified the presence of miRNA, mRNA and proteins in EVs and, more importantly, the ability to functionally shuttle their cargo into recipient cells, reinforcing the belief that EVs facilitate communication between cells [14, 15] and fuelling the idea of exploiting these vesicles for drug delivery applications.

1.2. Biogenesis, cargo loading and composition

Generally, EVs are categorized in three subtypes (i.e. exosomes, ectosomes and apoptotic bodies), based on distinct biogenesis pathways [16]. Apoptotic bodies are formed when a cell is dying *via* apoptosis, leading to blebbing and finally disintegration of the cell plasma membrane with partitioning of the cellular content in different membrane-enclosed vesicles. Hence, apoptotic bodies typically are larger particles (~0.5 - 4 µm) containing cytoplasmic organelles and fragmented nuclei [17]. Although some studies have reported a communication and biological function for these vesicles [18, 19], most research in this field discusses the smaller sized exosomes and ectosomes. Hence, apoptotic bodies are not considered when referring to EVs throughout this review. Exosomes (50 – 150 nm) and ectosomes (50 – 1000 nm) do not only show a partly overlapping size distribution but also their biogenesis pathways are very similar **(figure 1A)**. In both cases their formation is preceded by the assembly of membrane micro-domains composed of specific lipids (with an important role for ceramide) [20] and proteins followed by budding and subsequent fission or pinching off. The main difference between both formation pathways is the

location of the initial budding process. Indeed, ectosomes (also termed shedding vesicles or microvesicles) are released directly from the cell's plasma membrane. On the other hand, exosomes originate from the inward budding of early and late endosomes hence forming MVBs containing ILVs [21, 22]. Subsequently, the MVBs are transported to and fuse with the plasma membrane, requiring a dynamic interplay between members of the Rab and SNARE protein family, concurrently releasing the ILVs in the extracellular space [23-27]. Partly because both biogenesis pathway are analogous, to date there is no defined panel of markers to distinguish between both vesicle subtypes in a vesicular isolate. Nonetheless, a panel of generic markers (e.g. CD9, CD81, CD63, TSG101, *etc.*) was defined by the International Society of Extracellular Vesicles (ISEV) to indicate the presence of EVs in a sample [28].

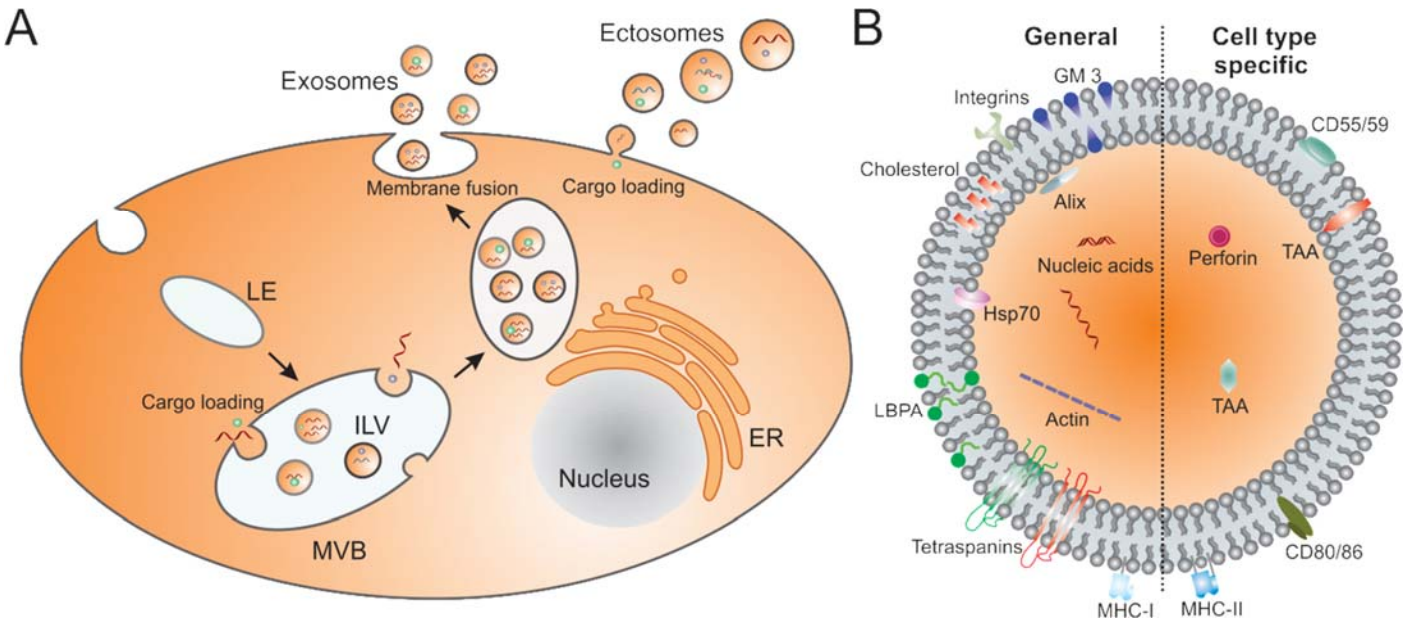

Figure 1. Schematic representation of EV biogenesis and architecture. [A] The biogenesis pathways of exosomes and ectosomes or shedding vesicles. Exosomes are formed by inward budding of the limiting membrane of early or late endosomes (LE) forming multivesicular bodies (MVBs) containing so-called intra-luminal vesicles (ILVs). Upon fusion of the MVBs with the cell membrane, the ILVs are released as exosomes in the extracellular medium. In contrast, ectosomes are released by direct budding from the plasma membrane. [B] The molecular

architecture of extracellular vesicles with some key general and cell-type specific molecular components. General: Tetraspanins (e.g. CD63, CD81), Alix, Heat shock proteins (e.g. Hsp70), major histocompatibility complex (MHC)-I, structural proteins (e.g. actin) , nucleic acids (e.g. miRNA, mRNA), integrins (type of integrin can be cell-type specific), lysobisphosphatidic acid (LBPA), cholesterol, ganglioside GM3 [29, 30]. Cell type specific: MHC-II, CD80, CD86 and complement shielding proteins CD55/59 (in DC) [31], tumor-associated antigens (TAA; e.g. GP100 in melanoma cells) [32], perforin (in natural killer T cells) [33].

Numerous papers report that the relative molecular composition of EVs differs distinctly from the producer cell. Lipidomic analysis showed an accumulation of cholesterol, sphingomyelin, glycerophospholipids and phosphatidylserine (PS) in EVs [34]. Certain membrane-associated proteins, for example many tetraspanins (e.g. CD9, CD81), appear enriched on the EV surface [35]. Finally, an array of reports show that specific mRNAs, miRNAs and other non-coding RNAs (e.g. t-RNA, Y-RNA, vault RNA, *etc*.) are enriched or underrepresented in EVs compared to their respective parent cells [36-41]. Based on these observations it is generally accepted that the composition of EVs is, at least partially, actively regulated by the parent cell [42], albeit that the mechanisms and associated key players regulating this cargo sorting remain largely elusive to date [38, 43-46].

The overall EV configuration (i.e. a lipoprotein shell encapsulating an aqueous core containing soluble proteins and nucleic acids) and part of the molecular composition (i.e. proteins and lipids required in the EV biogenesis) are common among EVs isolated from different cells [29]. However, some EV-associated molecules are unique for the producing cell type **(figure 1B)**. For example, MHC II is found on EVs secreted by antigen presenting cells (APC) [35, 47]. As another example, CD2, CD8 and CD56 were found in EVs derived from natural killer (NK) cells and not in EVs derived from platelets where the opposite holds true for CD41b, CD42a and CD61 [48].

Furthermore, it appears that the culture conditions not only influence the cellular phenotype but also the number and/or composition of the secreted EVs. For instance, hypoxia triggers cancer cells to release more CD63-positive vesicles [49] with a modified molecular composition and distinct effect on recipient cells [50]. Comparable observations were made for an altered extracellular pH [51] and the presence of stress-inducing molecules (e.g. lipopolysaccharide, H_2O_2, *etc.*) in general [52]. Besides the cellular microenvironment also the status of the cell influences the EV composition and downstream activity. Where mature DCs release pro-inflammatory EVs enriched in MHC II and ICAM-1 [53], EVs derived from DCs cultured in the presence of IL10, an anti-inflammatory cytokine, suppress the onset of inflammation in a mouse arthritis model [54]. The fact that phenotypic alterations in the parent cells are mirrored by the composition of the secreted EVs can be exploited for diagnostic purposes (**section 3.2.**).

The influence of the surrounding medium on the EV composition may have relevant clinical implications. For instance, Li *et al*. compared EVs derived from N2A neuroblastoma cells cultured both in serum containing cell medium or under starvation conditions, showing marked alterations in the protein composition [55]. Besides the changes in the composition of the EV itself, the presence or absence of serum proteins will likely also influence the protein corona surrounding the EVs . It is well documented that this corona strongly influences the extra- and intracellular (transfection) behavior of synthetic nanoparticles, including liposomes [56, 57]. Given the analogy between EVs and liposomes [58] it is conceivable that a protein corona will also impact the EV interactome and hence biological function. However, to date the influence of these parameters on the EV functionality has not been thoroughly investigated.

1.3. EV heterogeneity

Evidence is mounting that within the exosome and ectosome population many distinct vesicle subtypes exist. When a specific exosome release pathway (i.e. *via* Rab27a inhibition) was silenced, the secretion of only a specific set of exosome-related molecules (i.e. CD63, Tsg101, Alix and Hsc70) decreased whereas others (CD9 and Mfge8) were not affected [59]. This could indicate that different exosome subtypes exist originating from different biogenesis pathways. Additionally, Van Niel *et al.* showed a clear discrepancy in protein profile between EVs released from the apical or basolateral side of polarized epithelial cells [60]. Another report showed that vesicles isolated from conditioned cell medium and plasma by UC could be divided in two distinct populations by bottom-up density gradient UC. Both populations showed a different protein and nucleic acid composition, which correlated with a distinct biological effect on recipient cells [61]. To address this heterogeneity in more detail, more sensitive techniques have to be developed allowing single vesicle analysis. In this respect, a recent study by Smith and coworkers used Raman microspectroscopy to obtain a Raman spectrum, which can be regarded as a molecular fingerprint, on the single vesicle level. Following principal component analysis of the obtained spectra, these authors concluded that at least four types of vesicles with a clearly distinct molecular composition are released [62]. Conceivably, this is still an underestimation of the factual heterogeneity among EVs. Yet to date it is impossible to physically separate these specific EV subtypes as reliable markers are lacking. This implies that the composition of and functions attributed to EVs are likely the combined effect of multiple subtypes of vesicles. This notion further complicates the adoption of EVs in a pharmaceutical context as it is well possible that only a specific subfraction of vesicles induces a desired effect while others might entail off-target or even opposing effects.

1.4. EV purification protocols and stability

EVs represent only a fraction of the cell's secretome. Parallel to the growing research interest in EVs, different methods to isolate and purify EVs from conditioned cell medium or biological fluids have been developed. The most common approaches are listed in **table 1** and discussed further with a focus on their applicability in a pharmaceutical context. The predominant technique in the literature is based on differential centrifugation followed by UC, which is based on a difference in size and density between EVs and other components present in the respective medium [63]. It is important to note that many potential contaminants are co-purified using UC (e.g. lipoprotein particles, protein(-RNA) aggregates, *etc.*) [64, 65] and that the yield is relatively low (i.e. 10 – 20%) and dependent on the medium viscosity [66, 67]. Additionally, the impact of the high shear forces on the vesicle integrity are under debate. While some studies indicate no changes in the integrity of the EVs after UC [68, 69] others show subtle EV aggregation influencing the EV biodistribution [70, 71].

To increase both the vesicle yield as well as purity of the isolate, density gradient UC (iodixanol or sucrose) can be used [64, 72]. To underscore the superior separation resolution, it was shown that viral particles could be physically separated from EVs by using an iodixanol-based density gradient [73]. The major disadvantages of this technique are the long processing time, making this technology difficult to implement in a clinical setting [74], as well as the lack of knowledge regarding the effect of the density gradient media on the EV's functionality and the potential interference of gradient residuals with downstream processing [28].

Another method that was originally developed to concentrate viral particles [75], employs hydrophilic polymers (e.g. poly(ethylene glycol); PEG) and (high) salt concentrations to precipitate EVs. Although this method provides a high yield, which makes it interesting for small amounts of starting material or as a preparative concentrating step, it lacks specificity as many contaminants

(e.g. protein aggregates) are co-isolated [64]. Hence, interpreting downstream analysis of precipitated EV isolates, should be done with caution. Additionally, the PEG polymer is also present in the final isolate potentially shielding the EV surface and interfering with their functionality or downstream analysis [76].

Affinity-based capture of EVs has the potential to yield subpopulations with high purity. The most well-known affinity-based approach exploits protein recognition on the EV surface *via* antibodies (e.g. associated to beads, a polymer surface in a chip or a chromatography column) [77, 78]. Of note, this method requires knowledge of specific EV markers, which despite many years of research [79] are still difficult to identify. To circumvent the lack of specific markers, a more general approach was recently presented in which antibodies are substituted by heparin as it appears to have a general affinity for EVs. However, the cross-reactivity with other components present in the respective media is a possible concern [80]. Overall, affinity-based capture of EVs might be very useful in an on-chip diagnostic set-up using small sample sizes [81, 82]. Yet from a therapeutic point of view, when contemplating to use EVs as medicinal products, larger volumes will have to be processed, thus augmenting manufacturing costs. Additionally, non-destructive methods to elute the EVs from the capturing agents need to be co-developed.

Finally, several separation methods are being developed for EVs that exploit differences in size, including size-exclusion chromatography (SEC) and filtration [83]. SEC enables fast sample processing with high yield making it applicable in a clinical setting [70, 84]. However, sample dilution is inherent to the technique as well as co-purification of (a low percentage of) lipoproteins, which might limit the applicability of this technique in a diagnostic context [74, 83]. The main advantage of SEC is the mild conditions and hence retained EV functionality [85, 86]. On the other hand, sequential filtration steps can eliminate smaller and larger contaminants to concentrate EVs.

Yet a major disadvantage compared to SEC are the often high forces used (*via* air pressure or centrifugal forces) possibly compromising EV integrity. Moreover, sticking of EVs to the membrane filters might limit the yield [84]. In most cases filtration is used as a preparative step prior to one of the above mentioned techniques.

Combining different purification methods based on complementary principles will be imperative to process very complex samples (e.g. plasma). The sequential combination of techniques is already commercially available, e.g. with the exo-spin^{TM} system (CELL guidance systems). This approach merges an initial concentrating step using the precipitation method with subsequent SEC to enhance the sample purity. It is of note that the diversity of purification techniques used throughout the literature hampers unambiguous comparison of different studies. This is most pronounced in the field of biomarker discovery as it is clearly shown that different isolation methods might greatly alter the obtained RNA/protein profiles [64]. However, recently the amount of commercially available purification kits (e.g. ExoEasy Maxi kit, Qiagen; ExoPure^{TM} Immunobeads, Biovision; qEV, Izon Science), based on different technologies, is increasing which is beneficial for the reproducibility and ability to compare between studies using the same kits. Retained vesicle integrity and functionality is of pivotal importance in a therapeutic context. In this regard, a direct comparison between different purification strategies evaluating the therapeutic functionality of the obtained vesicle isolates would provide valuable information for pharmaceutical applications.

2. Therapeutic applications of EVs

2.1. Harnessing the intrinsic biological effect of EVs

As discussed above, EVs are composed of numerous potentially bioactive molecules (i.e. lipids, proteins, nucleic acids and carbohydrates) of which the relative composition is regulated by the producer cell. In this respect, it is no surprise that EVs have an intrinsic biological effect that

modulates the recipient cell's phenotype, which can be exploited in a therapeutic context [87]. These phenotypical alterations can be elicited by a receptor-ligand interaction at the cellular surface [88, 89] or at the luminal side of the (late) endosomes, thus triggering a downstream signaling pathway [90]. Alternatively, it is believed that EVs can fuse with cellular membranes (plasma membrane and/or endosomal membrane) and release their content in the cytoplasm [91] **(figure 2)**.

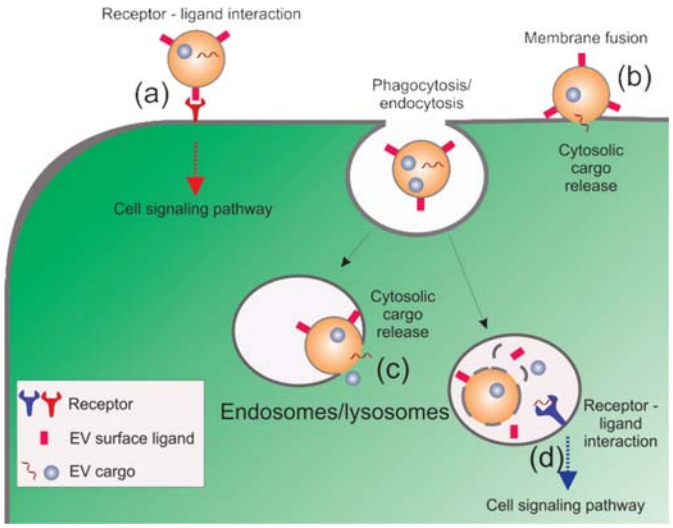

Figure 2. Mechanisms behind EV-mediated phenotypical changes in recipient cells. (a) The EV surface proteins/lipids can interact with receptors on the recipient cell's surface triggering an intracellular signaling pathway. Alternatively, (b) the EV cargo (e.g. proteins and nucleic acids) can be released in the cell's cytoplasm *via* membrane fusion with the limiting cell membrane or (c) with the endosomal membrane after initial internalization *via* phagocytosis/endocytosis. Finally, it is also possible that the (d) EVs release their content (after partial degradation) in the endolysosomes where it can trigger endosomal receptors (e.g. toll-like receptors).

As mentioned earlier, Raposo *et al*. showed that EVs derived from activated APCs could stimulate the immune system by presenting functional antigen-MHC complexes to T cells [9]. This observation was followed by many pre-clinical and clinical studies using antigen pulsed, DC-derived EVs as a cell-free alternative for cancer vaccination (**section 2.3.**) [92].

Another example of the use of EVs as a cell surrogate therapy are mesenchymal stem cell (MSC)-derived vesicles. MSCs are stromal cells with multipotent differentiation capacity and have been intensively investigated for their potential regenerative and immunosuppressive effects in many animal models and clinical trials. Although originally believed to be the result of MSC homing to and engraftment at injured tissues, it is now becoming increasingly clear that the biological effect of these cells is mainly attributed to their secretome, including EVs [93, 94]. In this respect, MSC-derived EVs have been studied in dedicated mouse models for their tissue-protective effects following acute kidney failure [93], myocardial infarct [95], liver injury [96] and neural injury after middle cerebrate artery occlusion [97]. Considering their immunosuppressive nature, MSC EVs are under investigation for a multitude of inflammatory conditions. For instance, in the field of auto-immune diseases, EVs shed by MSCs have shown to limit the pro-inflammatory response and induce a shift towards a beneficial regulatory T cell profile in type I diabetes [98] which is currently being investigated in a clinical setting (NCT02138331). As another example, MSC EVs are also successfully investigated in refractory graft-*versus*-host disease [99]. The exact mechanism behind the therapeutic effect of MSC-derived EVs remains largely obscure and is a topic of intensive investigation [100]. It is however known that stem cell EVs are enriched in signaling proteins, including cytokines, chemokines, interleukins and growth factors [101]. The use of EVs as a surrogate for cell-based therapies is intensively studied as it might entail some benefits. EVs are more resistant to freeze-thaw processes, are genetically stable making them a safer alternative to whole cells and they are likely less immunogenic allowing allogeneic therapy. Multiple comprehensive reviews have been published giving a more detailed overview of reported data on this topic [102, 103].

Next to their exploitation as surrogates for cell therapy, EVs from specific cell types have shown interesting features that can be exploited in a therapeutic context. For example, NK-cell derived EVs were shown to contain killer proteins (e.g. perforins), which are taken up by tumor cells and induce tumor cell death [33]. Adipose-derived stem cells (ADSCs) release EVs containing neprilysin (i.e. an Aβ-degrading enzyme), which lowered the Aβ-level secreted by N2A cells and thus might be a valuable therapy to investigate further in Alzheimer's disease [104].

It is of note that these reports have to be interpreted with careful consideration of the EV purification protocol used. Dependent on the selected method, non-EV contaminants can be co-isolated, possibly leading to observations being incorrectly attributed to EVs. Moreover, many reports focus on a specific component of EVs, e.g. small non-coding RNA such as miRNAs, often neglecting the true complexity of the EV composition in which lipids and proteins likely also play a key role [105]. As a result of this complexity, EVs can simultaneously interfere with different signaling pathways, leading to pleiotropic effects. For example, it was observed that EVs derived from immortalized cardiomyocytes (HL-1 cells) significantly altered the expression of 161 genes in fibroblasts (NIH/3T3 cells) after co-culture [106]. This complexity implies that the observed effects are likely very difficult to mimic by synthetic, single-API drug therapies. On the other hand, care should be taken that this intrinsic complexity does not impede the translatability of EVs into a viable pharmaceutical product [107].

2.2. Harnessing EVs as a drug delivery vehicle

2.2.1. Beneficial features of EVs as nanocarriers

As outlined above, EVs are involved in communication between cells owing to their ability to deliver biomolecules from one cell type to another, thereby crossing both extra- and intracellular

barriers. Based on this particular feature, EVs are also envisioned as biological nanocarriers for the delivery of exogenous therapeutic (macro)molecules. The encapsulation of drugs in nanoparticles (creating so-called nanomedicines) is a well-established approach to (1) modify the pharmacokinetics (PK) and biodistribution of the therapeutic cargo, (2) solubilize hydrophobic drugs, (3) protect the drug from the extracellular environment and (4) guide the therapeutic cargo across existing extra- and intracellular barriers. Both low molecular weight chemotherapeutics, but especially membrane-impermeable macromolecular drugs (e.g. nucleic acids and proteins) require nanocarriers to enhance their delivery across biological membranes. Unfortunately, many synthetic nanoparticles (including lipid- and polymer-based nanoparticles) demonstrate insufficient *in vivo* targeting to extrahepatic tissues and fail to merge (intracellular) drug delivery efficacy with biocompatibility [108]. Since the identification of EVs as nature's own intercellular communication tools, it is hypothesized that their Darwinian optimization could outperform conventional synthetic nanomedicines [109]. Indeed, EVs are believed to encompass many interesting features for drug delivery: (1) a proteo-lipid architecture that protects the encapsulated cargo, (2) their nanosize and specific composition minimizes recognition by the mononuclear phagocyte system (MPS) [110], (3) their patient self-derived nature mitigates activation of the adaptive immune system, (4) they contain specific lipids that help stabilize the vesicles in the blood circulation (e.g. GM3, sphingomyelin and cholesterol) and stimulate membrane fusion [51, 111] as well as surface proteins that have likewise been linked to membrane fusion in cell-cell and virus-cell interactions (e.g. CD9, CD81) [112, 113] and finally (5) EVs seem to possess intrinsic cell and tissue targeting properties [114].

2.2.2. Extracellular behavior of EVs

One of the main motivations to incorporate drugs into nanocarriers is to modulate their biodistribution and tissue targeting. Free drugs are often rapidly cleared from the body and show poor tissue selectivity, which can in part be remedied by their formulation into nanomedicines. Unfortunately, without appropriate surface modification (e.g. PEGylation), they are easily recognized by the MPS and rapidly cleared from the blood circulation, leading to predominant sequestration by liver and spleen and limiting extravasation in other tissues of interest. As EVs are abundantly present and stable in the blood circulation, it was speculated that EVs could have longer circulation times and mediate drug targeting to extrahepatic and non-lymphoid tissues. However, reports studying the PK of IV injected EVs described short half-lives (~2 minutes [115, 116] and ~20 minutes [117]) with predominant uptake by liver, lung, kidney and spleen, thus closely resembling the biodistribution of synthetic liposomes [70, 118, 119]. The elimination after IV injection occurs *via* hepatic and renal routes [117] in which MPS-associated macrophages seem to play a key role [118]. It is conceivable that this recognition is in part mediated by the exposure of PS at the external side of EV (subtypes) [120, 121]. It is of note that in these studies tumor- or HEK 293T-derived EVs have been used. For immature DC-derived EVs it was reported that they carry surface proteins (i.e. CD55 and CD59) inhibiting complement-mediated clearance [122]. Additionally, Whitehead *et al.* showed that EVs derived from malignant cells were far more prone to complement activation compared to non-malignant cells, which might help to explain some of the reported PK data [123]. Furthermore, also the selected purification protocol or the transfer of allogeneic EVs can potentially influence the EV's PK profile [70].

Despite the intrinsic targeting to APCs and limited circulation time often reported for EVs, it appears that a certain fraction is still able to home to alternative organs and tissues. For instance, it was shown by Hoshino *et al.* that the integrins present on the surface of tumor-derived EVs

determined the organs/cell types that are preferentially targeted [114]. Such observations rationalize the engineering of EVs with specific targeting moieties to enhance tissue or cell specific homing. One of the first engineered EVs was reported by Alvarez-Erviti *et al.* who equipped EVs from immature DCs with a Lamp2b-RVG targeting peptide, *via* genetic engineering of the producer cell with the respective plasmid construct, to enable delivery of siRNA across the blood brain barrier (BBB) [124]. The same targeting ligand was also used to shuttle liposomes over the BBB for the delivery of siRNA [125]. The BBB targeting enhancement was later quantified by Wiklander *et al.* to be around two-fold [119]. Nonetheless, the majority of the vesicles was still present in MPS-associated tissues (i.e. liver, spleen and lung) [119]. The fact that targeting ligands are providing modest benefits is likely the result of the short circulation time. On the other hand, the partial degradation of the RVG targeting peptide during EV formation might also contribute to this observation. Indeed, Hung *et al.* showed that when fusing a targeting peptide to the Lamp2b protein (a protein inherently present on the EV surface) it should be equipped with a glycosylation site to protect it against protease degradation by the producing cell. The unprotected Lamp2b-RVG targeting construct showed only marginally improved internalization by N2A cells bearing the nicotinic acetylcholine receptor compared to non-targeted EVs due to peptide degradation [126]. In analogy with reports on synthetic liposomes, efforts to simultaneously enhance the circulation time and confer specific targeting properties have also been explored for EVs. For instance, hydrophilic PEG chains were inserted in the EV lipid bilayer carrying targeting nanobodies at their distal end to both shield the EV surface from off-target interactions (leading to a prolonged circulation time) yet allow specific interactions with a targeted receptor [127, 128]. However, such approaches greatly alter the composition and behavior of EVs, both in the extracellular environment as well as following intracellular uptake, and the question is raised to what extent these approaches are advantageous over synthetic drug-loaded nanocarriers.

The ability to cross the BBB is an interesting and often referred to feature attributed to EVs. Although the RVG-targeting ligand associated to the EV surface in the previously mentioned studies likely plays a potentiating role [124], EVs derived from unmodified hematopoietic cells were also shown to cross the BBB. This event was reported to be rare, yet occurs more frequently under peripheral inflammatory conditions [129]. The mechanism behind this process remains to be elucidated. One hypothesis is based on transcytosis in which EVs are taken up by (apical) endocytosis by endothelial cells and are again released at the basolateral side following exocytosis [130]. A recent study compared four types of EVs derived from different brain cells (i.e. brain endothelial cells (bEND.3), glioblastoma A-172 cells, neural glioblastoma U87 cells and neuroectodermal tumor PFSK-1 cells) for their ability to deliver cytotoxic drugs over the BBB in an embryo zebrafish model. Only the bEND.3derived EVs were able to transfer their cargo into the brain, underscoring the existence of EV specificity [131].

Besides transferring cargo over the BBB, tumor targeting is another therapeutic application for which nanomedicines can provide a clear benefit. For this purpose, nanomedicines typically rely on the enhanced permeation and retention (EPR) effect to extravasate and accumulate in the tumor mass. Based on their small size, it is conceivable that EVs can also exploit this effect for anti-cancer treatment. However, such passive targeting of EVs to tumors gave rise to contradictory results. Smyth *et al.* IV injected EVs (60 µg) derived from the tumor itself but found very little amount in the tumor tissue (4T1 breast cancer and PC3 prostate cancer cells) compared to liver and spleen [118]. In contrast, Lai *et al.* found a marked signal of HEK-derived EVs (100 µg) in the tumor (Gli36 glioblastoma) alongside with the liver and spleen [117]. An example of successful tumor targeting by modified EVs was reported by Ohno *et al.* who observed a three-fold enhancement in the tumor tissue (HCC70 hepatocellular carcinoma) using EGFR-targeted (*via* the

GE11 peptide) EVs [132]. Comparable results were obtained with iRGD equipped EVs that bind to $\alpha_v\beta_3$ integrins in tumor tissue. Importantly, these vesicles, when loaded with doxorubicin, strongly reduced tumor growth in a MDA-MB-231 breast cancer cell model [133]. Inspiration for targeting is often obtained from viruses. For instance, HEK293-derived EVs have been modified with gp350 (i.e. a ligand for CD21 expressed on B cells and a component of the Epstein-bar virus (EBV) envelope), conferring the viral tropism to EVs for normal and leukemic B cell targeting [134].

Finally, altering the PK of EVs can also be done by changing the route of administration. Indeed, Wiklander *et al.* compared IV, SC and IP injection of HEK293T EVs showing a clear difference in biodistribution [119]. After footpad injection accumulation of EVs in the lymph nodes was reported [135, 136] and intranasal application showed an accumulation in the brain [137, 138] in which the delivered anti-inflammatory cargo (i.e. curcumin) could still be detected up to 12 hours after administration [137].

2.2.3. Intracellular trafficking of EVs

Especially when considering EVs for delivery of macromolecular therapeutics, which require delivery into the cell's cytoplasm (e.g. miRNA, mRNA) or even nucleus (e.g. pDNA), the ability of EVs to shuttle their cargo over the cellular barriers is of key importance.

Nanoparticles can employ distinct endocytic uptake pathways to gain access to cells. Numerous studies have investigated the mechanism(s) by which EVs are associated to and subsequently internalized by cells. Many different types of surface molecules, both EV- and cell-associated, have been identified as being involved in EV-cell contact (i.e. tetraspanins, integrins, proteoglycans and lectins) as comprehensively reviewed by Mulcahy *et al.* [139]. These interactions, possibly

preceded by surfing onto filopodia according to recent observations [140], mostly lead to cell uptake *via* one of the common endocytosis pathways (i.e. clathrin- and caveolin-dependent endocytosis, lipid raft-mediated endocytosis, macropinocytosis or phagocytosis) [105, 139]. It is also interesting to note that inhibition of a given pathway is almost never able to completely abrogate the EV uptake, hinting toward the involvement of multiple uptake mechanisms and/or reflecting EV heterogeneity [139]. In this regard, it would be an interesting strategy to also link the effect of uptake inhibitors to the induced phenotypical changes in recipient cells as this would help to elucidate which specific uptake pathway(s) leads to functional cellular release of the EV cargo.

As the interaction of EVs with cells likely involves multivalent ligand-receptor binding, it is reasonable to assume that they finally are trafficked to lysosomes for degradation [140]. Hence, delivery of drugs into the cell cytoplasm will require a mechanism that allows the EV cargo to escape the endolysosomal compartment. Also for synthetic nanomedicines the endolysosomal entrapment is one of the major hurdles for efficient cellular delivery of membrane impermeable drugs. The delivery efficiency of nanomedicines hinges on strategies to cross the endosomal barrier, such as the so-called proton sponge effect and/or lipid bilayer fusion [141]. As many of the effects mediated by EVs have been attributed to the functional delivery of miRNA and mRNAs, [87] this implies that (subtypes of) EVs might contain built-in mechanisms to stimulate endosomal escape. The most plausible theory describes back-fusion of internalized EVs with the endosomal membrane, thus releasing their content in the cytoplasm (**figure 2**). However, few reports directly demonstrated EV fusion with plasma-and/or endosomal membranes. Some studies labeled EVs with a self-quenching dye after which they were incubated with cells. An enhancement of fluorescence was indicative of dye dequenching and hence fusion of (a fraction of) EVs with cellular membranes [51, 91]. Alternatively, luciferin containing EVs were able to evoke a

luminescent signal after interaction with luciferase expressing cells, suggesting cytosolic delivery of the EV luminal cargo [91]. Whether this intracellular delivery process is linked to a particular receptor-ligand interaction or requires a specific proteolipid composition is currently unknown.

Alternative to relying on the intrinsic EV properties to obtain functional delivery, EVs have been modified with delivery-enhancing peptides. Temchura *et al.* decorated antigen-loaded EVs with a vesicular stomatitis virus G (VSV-G) protein to stimulate the cross-presentation of these antigens in recipient DCs [142]. The VSV-G protein promotes the fusion of lipid membranes at lower pH (i.e. ~6) and can thus drive destabilization of the endosomal/phagosomal membrane following internalization [143]. These authors showed that the VSV-G protein stimulated MHC I mediated antigen presentation and elicited an antigen-specific $CD8^+$ T cell response [142]. The previously mentioned RVG targeting ligand [119, 144, 145] and iRGD [133, 146] have also been reported to have membrane-destabilizing properties, possibly contributing to enhanced cytoplasmic delivery of the encapsulated cargo.

It is of note that not for all phenotypical effects EV internalization is necessary. Physiological effects attributed to EVs can be based on proteins and lipids present on the surface of EVs interacting with ligands on the target cell's surface triggering intracellular signaling pathways **(figure 2)** [88, 147-149] or *via* enzymatic activity present inside or on the surface of EVs [150].

2.2.4. Loading EVs with a therapeutic cargo

The clinical implementation of EVs as a viable drug delivery platform will require optimized methods allowing efficient loading with the drug of choice. As already indicated above, EVs have been evaluated as a drug delivery vehicle for a vast diversity of therapeutic cargos, including both small molecules (e.g. doxorubicin, curcumin,…) and macromolecules (i.e. RNA, DNA and

proteins). The strategies to incorporate these drugs into EVs can generally be divided in pre-and post-formation approaches [151]. In the former case, the therapeutic cargo is first loaded into the respective producer cell followed by its packaging into EVs during their biogenesis. For the latter approach, EVs are first purified from the producer cell's conditioned culture medium after which they are loaded with the therapeutic cargo *via* one of the methods represented in **table 2**.

In a pre-formation loading approach the endogenous sorting machinery of the cell is used to load the cargo into the EVs **(table 3)**. Loading of specific nucleic acids (siRNAs, miRNAs, mRNAs) into EVs can be accomplished by transfection of the producer cell with the respective cargo (e.g. miRNA) by lipofection [52]. A comparable approach, by incubating the producer cell (i.e. MSCs) with free paclitaxel, has also been evaluated. The paclitaxel-loaded EVs that were secreted by the MSCs induced an anti-proliferative effect on *in vitro* cultured adenocarcinoma cells [152]. Alternatively, the RNA of interest can be expressed in the producer cell *via* a plasmid vector encoding for the respective nucleic acid therapeutic (e.g. miRNA [153], siRNA [154], mRNA [155]). Unfortunately, such pre-formation loading approaches typically show limited loading efficiency and should be optimized for each selected producer cell type and cargo. In addition, one needs to anticipate that the selected cargo can influence the producer cell's functionality and viability, hence impairing the loading process. Although still largely elusive, our expanding knowledge on the endogenous cargo sorting machinery can be exploited to increase the drug loading efficiency by modifying the therapeutic cargo. In this respect, proteins can be equipped with a plasma membrane anchoring and oligomerization domain to stimulate EV loading [156]. Alternatively, proteins can also be sorted into EVs by creating a fusion construct containing the protein of interest linked to a protein that is inherently associated to EVs as has been done for EV targeting purposes **(section 2.2.2.)** [144, 157] or to fluorescently label EVs (e.g. CD63-GFP) [158].

Likewise, also for nucleic acids, evidence is mounting that by altering the nucleotide sequence also the sorting efficiency can be modulated. Bolukbasi *et al.* identified a specific sequence in the 3'-UTR region of mRNA strands that promotes its accumulation in glioblastoma-derived EVs. This ~25 nucleotide sequence contained a miR-1289 binding region and a CUGCC sequence. Incorporation of this so-called 'zip-sequence' in the 3'-UTR of a mRNA strand increased its packaging into EVs two-fold compared to the unmodified sequence. This enrichment could even be further enhanced when miR-1289 was overexpressed in the producing cell [39]. Regarding miRNA sorting, Koppers-lalic *et al.* discovered that 3'-uridylated miRNAs are enriched in human B cell-derived EVs [45]. Villarroya-Beltri and colleagues showed that miRNAs containing a GGAG sequence were overrepresented in primary T lymphoblast EVs. They suggest that this sequence is selectively recognized by the RNA binding protein heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNPA2B1) and subsequently drives incorporation into EVs [38]. However, these sequences could not be retrieved in miRNAs accumulating in colorectal cancer-derived EVs, implying the existence of distinct sorting pathways [159]. Although progress is being made, in general these sorting mechanisms remain vaguely defined to date.

In addition to the above mentioned loading approaches for small molecules and macromolecules, viral capsids (i.e. AAV) have been loaded in EVs thus creating so-called vexosomes. These hybrid vesicles are composed of viral particles coated with or associated to EVs. Vexosomes aim to merge the efficient transfection capabilities of the AAV and the immune-shielding properties of EVs to produce a potentially efficient and biocompatible delivery vehicle [160]. Indeed, EVs appear to protect AAVs from adaptive immune detection. Hence, vexosomes outperformed uncoated AAVs regarding *in vivo* transfection efficiency in the presence of neutralizing antibodies [161]. Moreover, the coating with EV membranes potentiated the AAV's ability to cross the BBB. The mechanism

behind this phenomenon is currently unknown [162]. Overall, the above mentioned features denote that this hybrid system is a potentially interesting therapeutic tool, combining the best of two worlds.

On the other hand, post-formation loading approaches attempt to load drugs in isolated and purified EVs. In this regard, the most frequently reported method, especially for hydrophilic membrane-impermeable components, is electroporation (EP). EP is traditionally used to introduce nucleic acids in cells using high-voltage electric pulses to create transient pores in the plasma membrane [163]. The group of Matthew Wood reported the first successful EP of siRNA into DC-derived EVs allowing functional delivery across the BBB in a mouse model of Alzheimer's disease [144]. Following this pioneering report, other research groups have shown comparable results for loading siRNA and even DNA strands up to a 1000 bp into EVs [164-168]. Besides nucleic acids, 5 nm superparamagnetic iron oxide particles and large proteins have been loaded in purified EVs using EP **(table 2)**. Despite the fact that different groups reported efficient cargo loading using this approach, the technique is under debate as it was shown that EP can induce the formation of large aggregates that co-precipitate siRNA and hence greatly overestimates the actual loading efficiency [169]. Since the publication of this report, several groups have tried to prevent this aggregate formation through the use of chelating agents (e.g. EDTA) [166] or membrane stabilizers (e.g. trehalose) [170, 171]. Nonetheless, even if transient pores would be formed in the EV membrane and aggregation can be prevented, given that EP likely relies on passive loading it can only be efficient in extremely concentrated EV isolates [169]. Other post-formation strategies that are being explored for hydrophilic molecules are also based on transiently destabilizing the EV membrane, including repeated freeze-thaw cycles, sonication, extrusion or saponin treatment **(table 2)**. These techniques have been evaluated for both small molecules (i.e. porphyrins) [172] as well as

macromolecules (i.e. the 240 kDa catalase enzyme) [138]. It is important to note that for the former methods, the integrity of the EVs can be significantly compromised [138]. One report also suggests that antibody-coated EVs derived from B1a cells can interact with and take up miRNAs from the environment by simple co-incubation and subsequently shuttle it into cells. However, the mechanism behind this post-formation miRNA loading as well as the generality of this loading approach remain to be elucidated [173].

For hydrophobic membrane-permeable molecules, simple co-incubation at ambient or elevated temperature are often sufficient to load EVs [131, 174]. Indeed, EVs derived from EL4 cells incubated with curcumin at room temperature were, after intranasal delivery, able to significantly delay brain tumor growth in the GL26 mice tumor model [137]. Curcumin-loaded plant EVs for example are currently under clinical evaluation in colon cancer (NCT01294072). An overview of other small molecules loaded *via* co-incubation is given in **table 2.** However, leakage of these therapeutics out of the vesicles in biological fluids (e.g. plasma) can limit their practicality.

As long as the fundaments of EV biogenesis and cargo sorting are not clear, pre-formation methods will suffer from limited efficiency. Indeed, when comparing both loading strategies for paclitaxel, the post-formation method yields ~21 mg/g EV [175] and ~7.3 mg/g EV [131] compared to ~2 µg/g EV [152] for the pre-formation method. Overall, loading hydrophobic small molecules in EVs is more straightforward and efficient. For post-formation loading of hydrophilic compounds, especially macromolecules, important progress still has to be made before efficient clinical application of EVs as drug delivery vehicles can be envisioned. In this respect, a generic post-formation loading strategy for siRNA was recently developed by exploiting the efficient hydrophobic interaction between cholesterol-conjugated siRNA (chol-siRNA) and the EV proteolipid surface [176]. Unfortunately, the EVs used in this study were not able to escape the

endolysosomal degradation pathway and hence failed to functionally deliver the siRNA in contrast to anionic fusogenic liposomes that were equally loaded with chol-siRNA. Moreover, the endogenously present miRNAs were not able to silence their respective target proteins which is in accordance with recent reports describing that (1) even the most abundant miRNAs found in EVs are secreted at a (low) ratio of 1 molecule per 100 vesicles [46, 177] and (2) internalized EVs are typically trafficked toward the lysosomes [140, 178]. Although this particular combination of EVs and recipient cells did not lead to successful EV-mediated drug delivery [176], it does not invalidate the concept of EVs as drug carriers as their interaction with cells might be highly specific. Therefore, a more in-depth biological understanding of the EV's delivery mechanism is urgently required, including cell type specificity, cellular uptake mechanism, intracellular trafficking and cellular cargo delivery..

2.2.5. Producer cell source selection

The choice of an adequate producing cell when aiming to exploit EVs as a drug delivery vehicle is of pivotal importance as it will define the PK behavior (i.e. the stability in the blood circulation and organotropism; **section 2.2.2.**) and the intrinsic biological effect (both physiological and pathological; **section 2.1.**) of the EV carrier. It has been suggested that MSCs form a sustainable source of EVs. MSCs produce high quantities of EVs and neither the EV yield nor their composition is altered by immortalizing the producer cell. Moreover, MSCs are known for their low immunogenicity making allogeneic applications possible [99, 179] (NCT02138331). However, it is also shown that MSC-derived EVs stimulate tumor vascularization and tumor growth, which might induce undesirable off-target effects [180]. Besides MSCs, immature DCs have also been proposed as an interesting EV source due to their low immunogenicity, immunosuppressive effects and the ease with which autologous sources can be obtained [109, 181].

As the field is moving closer to clinical applications, the concept of high vesicle yield with minimal production costs is of increasing importance. In this respect, research groups have started to focus on alternative sources of EVs. Grapefruit- and milk-derived EVs have been investigated as drug delivery vehicles [182-184]. Additionally, the idea of creating EV mimicking vesicles, e.g. by means of sequential extrusion of cells through micro-and nanoporous filters [185, 186] or by mixing synthetic components attempting to reproduce the most important EV characteristics [79, 187], is gaining interest. However, the latter approach is difficult to implement as long as the knowledge on which components are essential for EV functionality is lacking or incomplete.

2.3.EVs as vaccination platform

The first therapeutic application of EVs was based on the use of DC-derived EVs as a surrogate for DC-based anticancer vaccination, [10] as APC-derived EVs harbor both (antigen-loaded) MHC I and II as well as the necessary co-stimulatory factors, to directly trigger ($CD8^+$ and $CD4^+$) T cell activation [9, 47]. However, *in vivo*, DC-derived EVs likely interact first with endogenous DCs (*via* cell surface adhesion or intracellular processing), transferring their antigens to endogenous APCs and augmenting T cell activation [188, 189]. The use of DC-derived EVs for cancer immunotherapy has already been evaluated in phase I clinical trials for both melanoma [13] and non-small-cell lung carcinoma (NSCLC) [190]. Although DC-derived EVs exhibited an excellent safety profile, the therapeutic effects were limited with no substantial $CD8^+$ T cell response. Nonetheless, pre-clinical studies have shown that co-delivery of adjuvants could vastly improve the evoked immune response. In this respect, Chaput *et al.* reported the combination of DC-derived EVs with CpG (a TLR3 agonist) [191], Guo and colleagues combined DC-derived EVs with another TLR3 agonist, i.e. polyinosinic-polycytidylic acid (poly(I:C)) and cyclophosphamide [192] and Gehrmann *et al.* associated α-galactosylceramide (a iNKT stimulatory factor) to antigen-

loaded DC-derived EVs, which induced a potent NK, γδ T-cell innate immune response and enabled proliferation of antigen-specific T and B cells [193]. Currently, a phase II clinical trial in NSCLC patients is evaluating the combination of cyclophosphamide with DC-derived EVs (pulsed with a range of antigens and INF-γ) that showed an improved immune stimulatory capacity in pre-clinical studies [194] (NCT01159288).

As pointed out above **(section 1.2.)**, EVs derived from cancerous cells have shown to carry a panel of known (e.g. CEA, GP100, HER2, melan-A, PSMA) [32, 195] and likely to date unknown tumor antigens. This is not only of interest from a diagnostic point of view but also makes tumor-derived EVs, which have shown to outperform free antigens [193, 196] and whole tumor lysate [197, 198], an attractive candidate to evaluate as a cell-free vaccine. Building on these promising observations, a clinical trial has been conducted using EVs isolated from ascites fluid. Unfortunately, similar to the DC-derived EVs, the effect of unmodified EVs was unsatisfactory. However, when co-injecting GM-CSF as adjuvant, a pronounced anti-tumor cytotoxic T lymphocyte response was induced [199]. As for DC-derived EVs, tumor-derived EVs in preclinical reports benefit from the indirect antigen presentation by endogenous APCs. This can be stimulated by combining tumor-derived EVs with synthetic adjuvants [200] or using EVs derived from (genetically) modified tumor cells to enhance the presence of adjuvant-like components (e.g. heat treatment to enhance hsp70 in tumor-derived EVs [201] or genetically engineer tumor cells to release IL18 [202] or IL12 [203] in EVs). It appears that for both strategies of EV-mediated anticancer vaccination, vesicles have to be modified to enhance their immune stimulatory effect.

Despite the multitude of reports showing the potential of (adjuvant-modified) tumor-derived EVs as antigen delivery vehicles, caution should be taken as there is mounting evidence that tumor-derived EVs exhibit immunosuppressive characteristics. Indeed, besides antigens, the presence and

functional transfer of fasL [204], TGF-β [205] and NKG2D ligand [206] by tumor-derived EVs was also reported, all of which can blunt the activity of effector T cells. Moreover, some tumor-derived EVs are considered pro-metastatic *via* niche formation [114, 207], angiogenesis stimulation and extracellular matrix degradation (e.g. *via* presence of metalloproteinases) [208]. Additionally, providing a good source for tumor-derived EVs in a clinical context is not evident. The most elegant, easy accessible source is ascites fluid. However, only few tumors entail the accumulation of EVs in this biofluid [32]. Alternatively, EV mimics can be produced from cancer cell biopsies *via* sonication. Whether these vesicles have the same ability as natural EVs to evoke an anti-tumor immune response is not known [209]. To make use of tumor-antigen bearing EVs without the negative characteristics of tumor-derived EVs, a DNA vaccine (delivered *via* an adenoviral vector or EP) was developed that encodes a fusion protein comprising (the extracellular part of) a known tumor-antigen and an EV-associated protein (C1C2 domain of lactadherin or the gag protein). Expression of this fusion construct shuttles the associated antigens to the surface or lumen of secreted EVs, respectively [157, 210]. Nevertheless, this technology is limited to well-characterized antigens and would likely benefit from an additional immune modulator. Excellent dedicated reviews on the interplay between EVs and the immune system can be found in the literature [211].

Next to eukaryotic cells, also prokaryotic cells release vesicles in the extracellular environment, which are termed outer membrane vesicles (OMVs). It is interesting to note that the use of OMVs as vaccination tool against infectious diseases is currently the most advanced therapeutic application of EVs with different ongoing and completed clinical trials (up to phase IIIb; e.g. NCT01423084, NCT01478347, NCT02446743, …) and a selection of OMVs that have already reached market approval (e.g. Bexsero® and MenBvac® for serogroup B meningococcal disease).

For a comprehensive discussion on the use of OMVs as vaccination technology the reader is referred to Van Der Pol *et al.* [212].

3. EV Biomarker mining

3.1. An introduction to biomarkers

A biomarker can be defined as an objectively measured characteristic that indicates the medical state of the patient. Biomarkers can assist clinicians in making a reliable diagnosis and can be used as a clinical endpoint surrogate in clinical trials. For both applications it is critical that the correlation between disease and biomarker is well characterized and validated [213].

A reliable biomarker has to fulfill a number of prerequisites. First, a biomarker needs to be specific, a feature with which many known biomarkers struggle (e.g. prostate specific antigen (PSA) often gives false positives for benign prostate hypertrophy) [214]. Also, it is of critical importance that a biomarker is robust and valid meaning that under all given conditions a correlation exists between the biomarker and the disease. In this respect it is important to have a clear understanding of the role of the biomarker in the pathophysiology of the disease. Ideally, biomarkers should be predictive, indicating that the quantity of the biomarker can be (positively or negatively) correlated with the severity of the disease. Furthermore, it is of interest that the biomarker is easy accessible, thereby minimizing the burden for the patient. Finally, the sensitivity of the biomarker (or diagnostic assay to quantify the biomarker) will determine the extent to which early diagnosis is feasible [213].

Parallel with the emergence of personalized medicine, the importance of adequate biomarkers is further increasing. Personalized medicine can provide a significant benefit for diseases exhibiting a strong inter-patient pheno- and/or genotype heterogeneity as is the case for many tumors [215].

Therapies that are tailored towards a specific phenotype (e.g. Herceptin® for Her2 positive breast cancer patients) are often developed in parallel with a biomarker assay, which enables the clinician to select patients who are eligible for the respective therapy [216].

3.2.EVs as biomarker

EVs can be regarded as a stable and easy accessible fingerprint of the parent cell [217]. Indeed, the EV composition will depend on the type and even status of the producer cell [218, 219]. As EVs are easily secreted by the large majority of cell types in the human body, they can be retrieved from all bodily fluids [220]. EVs have been isolated from e.g. urine [11], plasma [26], semen [25], nasal secretion [24], breast milk [221], the aqueous humor of eyes [222], cerebrospinal fluid [223], peritoneal fluid [224], bronchoalveolar lavage [225]. Depending on the respective disease for which the biomarker is being developed, an accessible biofluid should be considered in which the EVs of interest are likely the most concentrated and a liquid biopsy can be easily obtained. Moreover, due to their liposome-like architecture, EVs protect their cargo against the harsh environment present in many of these media providing a more stable, hence reliable biomarker source compared to naked RNA or proteins in e.g. blood.

EVs have been linked to a plethora of (patho)physiological processes. They are involved in maintaining cellular homeostasis but have also been linked to for example cancer progression. Glioblastoma-derived EVs have shown the ability to spread oncogenic transformation by transferring the oncogenic form of the epidermal growth factor receptor (EGFRvIII) to surrounding cells [226]. These EGFRvIII expressing vesicles were also detected in the serum of 7 out of 25 glioblastoma patients and have been proposed as biomarker source [12]. Additionally, tumor-derived EVs have shown the ability to promote cancer growth by inhibiting cancer-specific immune recognition (**section 2.3.**) [88, 227]. Moreover, EVs are also involved in the metastasis of

tumor cells as they are believed to prepare a pre-metastatic niche at a secondary tissue or organ (the seed-and-soil hypothesis) [114, 135, 207]. The fact that EVs play such an important role in the process of tumor formation strengthens the validity and robustness of their use as biomarker in cancer detection. Besides cancer, EVs are also exploited by viral particles (e.g. HIV, Epstein-bar, hepatitis C) to mediate their spread, making EVs valuable tools to detect viral diseases as well [228]. Furthermore, EVs are associated with neurological, metabolic, cardiovascular and kidney conditions and are therefore also proposed as biomarkers for these diseases [229-231].

In the literature many different clinical samples have been shown to contain EV-associated biomarkers with diagnostic/prognostic value or disease monitoring potential. In this respect, the EV concentration present in serum of tumor-bearing patients was shown to be increased compared to healthy controls [217, 232-235]. The EV protein abundance also has prognostic value as it was observed that patients with stage III melanoma with a high EV-associated TYRP2 protein burden showed increased risk of disease progression [207]. Moreover, following resection of the primary tumor, the EV concentration markedly decreased indicating its correlation with tumor presence [235]. However, relying solely on EV concentration lacks specificity as the same observation was made for distinct cancer types [217, 232-235] and, importantly, for non-disease stimuli (e.g. physical exercise [236]). Furthermore, early diagnosis of many cancers will not be possible. Therefore, it is of outstanding interest to look in more detail to the EV cargo (i.e. proteins, miRNA, mRNA,…) as they provide an easy accessible window to monitor the status of the respective producer cell **(section 1.2.)**. In this respect, the exploitation of comparative omic-studies is fundamental for the detection of new biomarkers. For instance, it was revealed that a panel of eight EV-associated proteins were upregulated in the urine of patients with bladder cancer compared to healthy subjects [237]. Likewise, miRNA profiling of plasma-derived EVs identified a panel of

four tumor-specific miRNAs of potential use in a screening test for lung carcinoma [238]. A comprehensive review on this topic was recently issued by An and colleagues [239].

Isolating EVs from a liquid biopsy prior to molecular analysis enhances the sensitivity (compared to whole blood/urine analysis) as highly abundant serum/plasma proteins (e.g. albumin) and urine proteins (e.g. Tamm–Horsfall glycoprotein) are removed [240]. It is estimated that less than 0.01% of the proteins present in plasma are EV associated [241]. It is important to realize that in biological fluids, in general the vast majority of EVs are derived from healthy cells. In this respect it is reasonable to speculate that the sensitivity and specificity can be further enhanced through isolation of cell- or tissue-specific EVs prior to a biomarker assay. Such an approach was explored by Taylor and colleague who isolated EVs from plasma by antibody-based capturing (using anti-EPCAM) and subsequently analyzed the miRNA profile in this tumor EV enriched population. They could show that the miRNA profile closely resembled that of the original primary tumor cell. The combination of EPCAM-based EV capture and downstream miRNA quantification could hence be used to distinguish between healthy patients and patients at different stages of ovarian cancer [233]. Another example of the importance of an upstream EV selection was provided by Shi *et al.* who measured α-synuclein levels in plasma of healthy individuals and patients suffering from Parkinson's disease. When analyzing α-synuclein levels in plasma using the total EV population, no significant difference could be observed between both groups. However, when the quantification was performed on plasma EVs positive for L1 cellular adhesion molecule (L1CAM), which is primarily expressed in the central nervous system, the α-synuclein levels were significantly enhanced in Parkinson's disease patients [242]. However, specific cancer markers are not always known or present on the EV surface. Additionally, population assays neglect an additional level of complexity conferred by the specific composition of individual vesicles, which

can provide relevant supplementary information. Therefore, techniques that allow analysis on the single vesicle level are of great interest [243]. However, for diagnostic purposes such an approach will require screening of large amounts of vesicles as 'diseased' EVs are rare among the total isolated EV population. One promising approach relies on modifying flow cytometry equipment/protocols to detect single, nanosized EVs [244-246]. However, to date FACS is not able to detect the lower size range of EVs and requires antibodies (and hence also knowledge of a particular disease marker) to phenotype EVs. Unfortunately, antibody-independent techniques that combine single vesicle sensitivity and high acquisition speed are scarce. One alternative strategy recently explored relies on surface enhanced Raman spectroscopy (SERS), which provides a quantitative molecular fingerprint of single EVs with a minimal acquisition time (500 ms) (**figure 3**). Based on the obtained Raman spectra, EVs derived from erythrocytes and melanoma cancer cells could be distinguished [247].

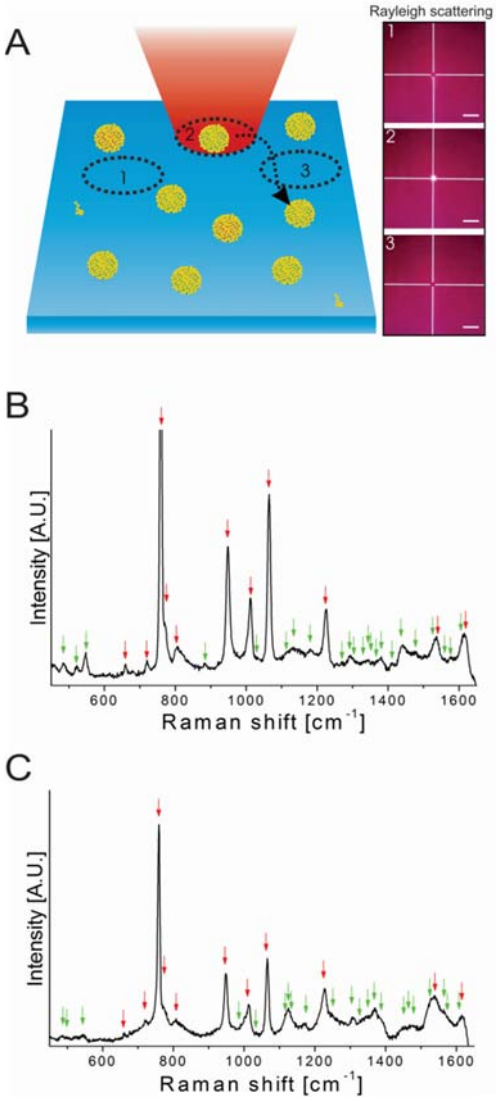

Figure 3. Surface Enhanced Raman Spectroscopy (SERS) characterization of single EVs. [A] Enhancement of the Raman signal was obtained by decorating individual EVs with a AuNP-based nanoplasmonic SERS substrate. Raman spectra were recorded by moving the focal volume through the sample. The location of a AuNP-coated EV was determined by Rayleigh scattering (as depicted on the right insert of panel A, image 2). Using this approach, SERS spectra were obtained from [B] B16F10 melanoma-derived EVs and [C] erythrocyte-derived EVs. Red arrows in the displayed representative spectra originate from the AuNP coating material, while green arrows mark EV-related peaks. Partial least squares discriminant analysis on the obtained spectra allowed to distinguish between both vesicle types in a mixture, demonstrating the potential of single vesicle SERS fingerprinting in a diagnostic context. Reproduced with permission from ref.[247]. Copyright 2016 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

3.3.Perspectives

The wide-spread interest from both academia and industry in exploiting EVs in a diagnostic context is evident from ongoing and completed clinical trials (NCT02702856, NCT01779583, NCT02147418, NCT01860118, NCT02439008, NCT02464930, NCT02662621) and extensive investments from the pharmaceutical industry (e.g. Exosomedx, Exosome sciences, Codiak Biosciences, Hansabiomed, *etc.*). These investments are accompanied by a multitude of filed patents claiming technical solutions for the purification and/or readout of this new type of biomarker source. A first diagnostic test (the ExoDx Lung (ALK) by Exosome Diagnostics), based on detecting a specific NSCLC-associated mutation present in exosomal RNA became commercially available at the beginning of 2016 [248].

Nonetheless, various issues still hamper the full exploitation of their biomarker potential. For instance, the lack of standardized purification protocols counteracts reproducibility and strongly influences biomarker identification. Due to this lack of consensus, to date no specialized EV-biobanks, where a specific biofluid/biopsy sample can be correlated to the patient's medical record, are established [249]. Moreover, elaborate purification protocols preclude fast screenings and hence restrain investigation and validation in large patient cohorts. Besides the purification protocol, also other parameters, among which the sample collection procedure (e.g. the type of anticoagulant during blood collection [250]), processing time and sample store conditions [65, 86] can influence the outcome of biomarker identification studies [251]. In response to this unmet need, an ISEV position paper was issued describing guidelines on how to handle different biological fluid samples and emphasizing the importance of a comprehensive experimental description to enhance reproducibility [252].

4. General conclusion

Inspired by their involvement in many (patho)physiological processes and their role as nature's own intercellular transport vehicles for biomolecules, a multitude of therapeutic and diagnostic applications have been explored for EVs.

To date, EVs have been successfully exploited as biological nanocarriers for synthetic drugs ranging from small molecule chemotherapeutics to macromolecular siRNA, proteins and mRNA in various preclinical studies. However, clinical translation will essentially depend on substantial improvements in cost-effective EV isolation methods, improved drug loading techniques and more detailed knowledge on EV composition, heterogeneity and inherent biological effects. Additionally, a knowledgeable assessment of the value of EVs as drug delivery vehicles will require a direct comparison between EVs and current state-of-the-art synthetic and viral delivery vehicles.

The complex composition of EVs conceivably correlates with off-target effects. On the other hand, this inherent complexity conferred by the many bioactive components associated to EVs enables them to induce potential beneficial effects likely challenging to mimic with therapeutic formulations containing a single drug. In this respect, MSC-derived EVs have been investigated in the field of regenerative medicine, auto-immune diseases and other inflammatory conditions as a safer alternative to whole cell therapeutics. EVs derived from both antigen-pulsed DC and tumor cells have been tested extensively for vaccination purposes. Despite the fact that the current clinical data show limited effect, pre-clinical reports indicate that modifications (e.g. co-delivery of an adjuvant) can further stimulate the evoked immune response. Nonetheless, it is important to note that safety concerns on the use of tumor-derived EVs are raised as many reports have linked EVs released by tumor cells to disease progression and metastasis. Here, ample attention should be given to further optimize EV purity and characterization protocols.

Finally, EVs have great potential to be harnessed in a diagnostic, prognostic and treatment monitoring context. EVs form a reliable and easily accessible window on the physiological status of the parent cell. They contain a vast amount of molecular information, which can be extracted by downstream proteomic, transcriptomic, miRNomic and lipidomic analysis, the feasibility of which has recently been underpinned by the first EV-based diagnostic test entering the US market. To galvanize further development of EVs as biomarkers, again fast, efficient and standardized purification protocols in combination with sensitive quantification methods will be essential.

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Table 1. Overview of the most used EV isolation methods.

Method	Principle of separation	Purity	Integrity	Disadvantages	Advantages
Ultracentrifugation (UC)	Size and density	Medium	High shear forces might affect EV integrity/functionality	Relatively long procedure (~4-5 hours) The yield is drastically reduced when the viscosity of the samples is high (e.g. plasma > serum > celmedium > PBS) [67]	Most used technique throughout the literature
Density gradient UC / sucrose cushion UC	Size and density	High	Mild forces	Long procedure (~18 hours) Effect of the gradient forming molecules on the EV functionality is unknown	Previously used in clinical settings [13, 190, 199, 253]
Precipitation	Salting out EVs using a PEG/salt solution	Low	Mild forces	Low purity The PEG chain might envelope the EVs, possibly interfering with their functionality	Applicable for large volumes Experience from the viral field Previously used in a clinical setting (as an EV concentration step prior to UC) [99]
Affinity capture	Binding of EV surface components	High	Mild forces	Expensive (if antibody based) EV elution might damage surface proteins and functionality	This method has the highest potential to physically separate different EV (sub)populations. However, due to the lack of specific markers for EV subtypes to date, this method is most frequently applied in the diagnostic field
Size exclusion chromatography (SEC)	Size	Medium to high	Mild forces	The final EV isolate is diluted	Chromatographic methods (e.g. SEC and IEC) are often used in clinical settings (e.g. to purify monoclonal antibodies)
Sequential filtration	Size	Unknown	Risk of modifying the original EV architecture due to extrusion	Sticking of EVs to the filter membrane lowers the yield	Useful as a pre-process concentration step. Previously used for this purpose in clinical settings [13, 253]

Table 2. Post-formation loading of EVs

Method	Cargo	Efficiency	Remarks	Ref
EP: • M^+ electrodes (400 V; 125 µF) • Buffer: Optiprep™; neutral pH	siRNA	25% of the total RNA	The reported RNA exceeds maximal theoretical loading based on the total vesicular volume% and assumption that loading is a passive process [169]	[124]
EP: • M^+ electrodes (150 V; 100 µF) • Buffer: Cytomix buffer	siRNA	90% of the total number of beads carrying EVs is positive for siRNA		[168]
EP: • M^+ electrodes (400 V; 125 µF) + polymer electrodes (variable settings) • Buffer: Optiprep™; neutral pH, EDTA containing buffer, acidic pH	siRNA	No significant encapsulation	Shows the formation of aggregates during the EP process and highlights the importance of adequate controls when using EP as a loading method.	[169]
EP	miRNA	No significant encapsulation reported		[132]
EP: • M^+ electrodes (0.75 kV/cm) • Buffer: Trehalose containing buffer	5 nm superparamagnetic iron oxide nanoparticles	0.40-0.45 µg iron/µg EV		[170]
EP: • M^+ electrodes • Buffer: PBS	70 kDa Dextran / Saporin	0.4% and 0.5% of the total dextran and saporin, respectively.	Required Lipofectamine LTX and GALA fusogenic peptide for functional delivery	[254]
Co-incubation (T=37°C)	miRNA (miR-150)	N.R.	Indirect proof of successful delivery is provided *via* restoration of the effect when using miR-150 knockout EVs	[173]
Co-incubation + 0.01% saponins, *freeze/thaw, *sonication, *extrusion	Catalase (240 kDa)	~15 – 25% of the added catalase	*Indicated technique entail significant alteration of the EV structure	[138]
Co-incubation (+ 0.01% saponins), hypotonic dialysis	Porphyrins	Up to ~$2.5x10^{15}$ molecules/EV		[172]
EP: • M^+ electrodes (350 V and 150 µF) • Buffer: non-specified electroporation buffer	Doxorubicin	20% of the added doxorubicin	EVs equipped with a targeting ligand (i.e. iRGD associated to LAMP2) – non targeted EVs were not functional	[133]
Co-incubation (T=22°C – 5 min)	Curcumin	2.9 µg/µg EVs	IP injection	[174]
Co-incubation (T=22°C – 5 min)	Cucumin / JSI-124		Intranasal administration	[137]
Co-incubation (T=37°C – 2 h)	Doxorubicin / paclitaxel	132 ng/µg and 7.3 ng/µg, respectively		[131]

EV-liposome mixing followed by freeze/thaw cycles	Lipids (-PEG)	/	Alteration of the EV membrane composition	[128]
EV-micelle mixing followed by elevation of temperature (40°C)	Lipids (-PEG) (-nanobody®)	/	Incorporation of targeting nanobodies® + PEGylation for an enhanced *in vivo* circulation time	[127]

EP electroporation; M^+ metal electrodes; PBS phosphate buffered saline; GALA: a pH-sensitive fusogenic peptide; IP Intraperitoneal; PEG poly(ethylene glucol); N.R. not reported

Table 3. Pre-formation loading of EVs

Cargo	Producer cell	Method of producer cell transfection	Efficiency	Target cell/Functional delivery?	Remarks	ref
Mir-143 and mir-143BP[1]	THP-1	Lipofection: LipoTrust™ EX Oligo + miRNA	Estimation: 0.2-0.25% of the stabilized miRNA present in the cell	No functional delivery reported	Differentiation of THP-1 cells into macrophages further stimulated miRNA secretion in EVs / immune-EM confirmation of miRNA presence	[255]
mRNA	HEK-293T	Lipofection: Lipofectamine 2000 + plasmid (+3' UTR zipcode sequence)	No absolute values reported per EV (~2 fold increase *versus* non-modified)	No functional delivery reported		[39]
mRNA (CD-UPRT) mRNA)/respective protein	HEK-293T	Lipofection: Lipofectamine 2000 + plasmid (equipped with a strong promoter i.e. cytomegalovirus promotor)	No absolute values	HEI-193 cells	Used as enzyme to functionalize a small molecule prodrug/sucrose gradient to confirm EV association	[256]
miRNA	HEK293	Lipofection: HiPerFect reagent + plasmid (final concentration 50 nM)	No absolute values	HCC70 cells	GE11 peptide for EGFR targeting	[132]
miR-143, miR-146a, miR-155	HEK293/COS-7	Lipofection: LipofectamineLTX + plasmid overexpressing the respective pri-miRNA	2.57%, 15.6%, 1.38% (percentage extracellular *versus* intracellular)	COS-7	EV association confirmed using RNAse treatment/using the neutral sphingomyelinase inhibitor GW4869	[153]
mRNA: Cre recombinase mRNA(+protein?) [2]	MDA-MB-231 mammary tumor cells	Lipofection: lipofectamine 2000 + plasmid	No absolute values	MCF-7 and T47D mammary tumor cells	Included delivery over long distance *in vivo*	[155]
(Cy3-tagged) miR-223	Macrophage (IL-4 activated)	Lipofection: X-tremeGENE siRNA transfection reagent + miRNA	No absolute values	Breast cancer cells (SKBR3)		[257]

(FITC-tagged) miR-150	THP-1 cells	Lipofection: lipofectamine 2000 + pre-miRNA (400 pmol per 60 mm dish)	0.002 pmol miRNA per µg EV (protein concentration)	HMEC-1 cells		[52]
siRNA	L929 cells	Lipofection: lipofectamine 2000 + siRNA duplex (60 pmol per 10^5 cells)	0.001pmol siRNA per µg EV (protein concentration)	S180 cells	0.4 pmol siRNA per mouse	[258]
Paclitaxel	MSCs (SR4987 cells)	Incubation of producer cell with 2000 ng/ml paclitaxel for 24h	2.03 ng paclitaxel/mg protein	CFPAC-1 (i.e. a paclitaxel sensitive adenocarcinoma cell line)	EVs loaded with paclitaxel show an anti-proliferative effect	[152]
Adeno-associated virus (AAV)	HEK 293T cells	Calcium phosphate transfection of plasmids coding for different AAV components	0.01% - 0.2% of the produced AAV are associated to EVs (depending on the AAV subtype)	U87 and 293T cells		[160]
TyA-GFP as model protein	Jurkat T-cells	Electroporation of the respective plasmid	>10 fold increase compared to unanchored	/.	Targeting proteins to EVs *via* membrane anchors[3] and the TyA-oligomerization domain	[156]
siRNA (*via* plasmid)	HEK 293T	Lipofectamine 2000 + plasmid	~0.15 pmol/µg EV	Neuro2A	RVG targeting (*via* fusion to the LAMP2 protein)	[154]
Iron oxide nanoparticles	THP-1	Incubation in serum free medium		SKOV-3 cells		[259]

[1]Bp: an aromatic benzene-pyridine analog was added to the 3′-overhang region of the RNA strand (higher nuclease resistance). [2] part of Cre-LoxP system to visualize functional protein/mRNA delivery to recipient cells. [3] e.g. myristoylation tag or PIP2-binding domain.

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