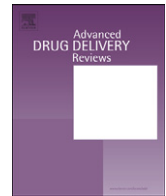




Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



Virus-modified exosomes for targeted RNA delivery; A new approach in nanomedicine[☆]



Danijela Koppers-Lalic, Marye M. Hogenboom, Jaap M. Middeldorp, D. Michiel Pegtel^{*}

Department of Pathology, Cancer Center Amsterdam, VU University Medical Center, de Boelelaan 1117HV Amsterdam, The Netherlands

ARTICLE INFO

Article history:

Accepted 8 July 2012

Available online 20 July 2012

Keywords:

Exosomes
MicroRNA
Genetic-transfer
Virus
Gene-therapy

ABSTRACT

A major goal in biomedical research is to clinically reverse the cause of disease rather than treating the symptoms. Gene therapy has the potential to meet this goal and the discovery of RNA interference (RNAi) has led to a new class of highly selective therapeutics. However, initial enthusiasm is reduced due to safety concerns associated with virus-based delivery vectors that are used for *in vivo* delivery.

Viral vectors for siRNA delivery into target cells are used because of their high target specificity and delivery efficacy (endosomal escape). Recent discoveries suggest that a specialized form of nano-sized lipid vesicles called exosomes can incorporate and transport functional RNAs into target cells and may serve as an attractive alternative. Evidence is accumulating that most pluricellular organisms sustain exosome-based communications via inter-cellular exchange of mRNA and miRNAs between cells. We discovered that viruses have found ways to exploit this communication pathway and we argue here that adaptations of exosomes imposed by viruses maybe exploited for superior delivery of RNA *in vivo*. We discuss recent discoveries in exosome biogenesis their physical properties, targeting and delivery strategies and how the knowledge of exosome production in virus infected cells could propel their entry into clinical settings.

© 2012 Elsevier B.V. All rights reserved.

Contents

1. Introduction	348
2. MVBs and the molecular machineries that regulate exosome biogenesis	349
2.1. Properties of exosomes	350
2.2. Endosomes/endocytic pathway	350
3. Viral factors in cell targeting and entry	351
3.1. Viral factors can be exploited for targeting therapeutic vesicles	352
4. Viruses manipulate exosome composition and production	352
4.1. Virus modified exosomes deliver functional genetic material	353
5. Exosomes current and future perspectives	353
Acknowledgments	354
References	354

Abbreviations: ABC, ATP-binding cassette; AGO, Argonaute; BACE1, β -site APP-cleaving enzyme 1; BBB, blood brain barrier; Dex, dendritic cell- derived exosome; EBV, Epstein–Barr virus; EE, early endosome; ESCRT, endosomal sorting complexes required for transport; HCC, hepatocellular carcinoma; ILV, intraluminal vesicles; imDC, immature dendritic cell; imDex, immature dendritic cell-derived exosome; LCL, latency type III lymphoblastoid B cell line; LE, late endosome; LMP1, latent membrane protein 1; mDex, mature dendritic cell-derived exosome; MDR1, multidrug resistance protein 1; miRNA, microRNA; mRNA, messenger RNA; MVB, multivesicular body; P-body, processing body; PEG, poly(ethylene glycol); piRNA, piwi-interacting RNA; RISC, RNA-induced silencing complex; RNAi, RNA interference; siRNA, small interference RNA; VLP, virus-like particles; VSV-G, G protein of vesicular stomatitis virus.

[☆] This review is part of the *Advanced Drug Delivery Reviews* theme issue on "Exosomes: a key to delivering genetic materials".

^{*} Corresponding author. Tel.: +31 204444052; fax: +31 204442964.

E-mail address: d.pegte@vumc.nl (D.M. Pegtel).

1. Introduction

Successful implementation of genetic materials as new therapeutic tools relies to a large degree on the appropriate delivery system. The efficiency of delivery by current methods still remains too low for many clinical applications or has raised serious safety concerns in experimental clinical trials. Therefore, new delivery agents are needed that combine a high degree of target specificity and efficient functional delivery with minimal immunogenicity. Ideally, such carriers could be easily loaded with a therapeutic cargo of choice e.g. nucleic acids, protein or drug. The first step towards efficient therapeutic delivery of siRNA or microRNAs (miRNAs) upon incorporation into the carrier vesicle is favorable

association with and internalization of the transport carriers, by the target cell. Depending on their surface characteristics and size, these vesicles are taken up by recipient cells via different endocytic routes, including clathrin- or caveolae-dependent endocytosis, macropinocytosis and phagocytosis. However, not all routes of delivery and uptake will ultimately lead to effective functional cargo release in the desired intracellular compartment. RNAi-mediated therapeutics via naturally produced nano-vesicles known as exosomes have received much attention because of their apparent ability to incorporate functional miRNAs and to deliver them to target cells [1]. However, how these vesicles select for specific recipient cells *in vivo* remains incompletely understood. Interestingly, exosomes when released by certain virus infected cells, appear to be equipped with selected viral products that modify and potentially enhance their physiological function. Such exosomes may thus hold critical advantages that could be exploited for siRNA and/or miRNA targeting and delivery *in vivo*. Thus knowledge on viruses and how they exploit exosomes may aid in the engineering of potent nano-vehicles for specific and efficient delivery of genetic material.

2. MVBs and the molecular machineries that regulate exosome biogenesis

During maturation of late endosomes (LE) the limiting membranes of endosomes start to bud inward resulting in the formation of isolated intraluminal vesicles (ILV) approximately 100 nm in size. These specialized type of endosomes are known as multivesicular bodies (MVB) and the ILVs function presumably as dynamic platforms for the selective

sorting of ubiquitinated and non-ubiquitinated cargo destined for either degradation in lysosomes or recycling. After fusion of MVBs with the lysosome, the cargo is degraded by the acidic environment of the formed endolysosome [2]. However, not all ILVs are delivered to the lysosomes for degradation. Some ILVs escape degradation and instead are secreted into the extracellular environment upon fusion of the limiting membrane of the MVB with the plasma (see Fig. 1) [3] that allows secretion of the ILVs as exosomes. Recent studies indicate that these extracellular membrane vesicles of endocytic origin have interesting characteristics in common with enveloped RNA viruses [4]. Indeed, like retroviruses, exosomes incorporate genetic material such as microRNAs (miRNA) and are able to deliver these to neighboring and distal cells. Moreover their RNA content is delivered to specific intracellular sites of the recipient cell where ‘RNA-induced silencing complexes’ (RISC) are situated that have an important role in epigenetic gene regulation [5]. These important key features of exosomes prompted a high interest of the drug delivery community because these properties may be exploited for drug delivery purposes.

The multivesicular body (MVB) is important for the sorting of molecules for the degradation pathway or for exosome secretion [6]. Ubiquitin attached to proteins is a targeting signal to direct cargo into the ILVs. In the lysosome the cargo will be degraded [7]. However, cargo can also be excluded from these ILVs and can be recycled or transported to other sites or cellular compartments [6]. Endosomal sorting complexes required for transport (ESCRTs) are involved in the trafficking of the cargo from endosomes to lysosomes for degradation. Four distinct ESCRTs are known, namely ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III. ESCRT-0, -I and -II recognize the ubiquitylated cargo in the MVB that is marked for

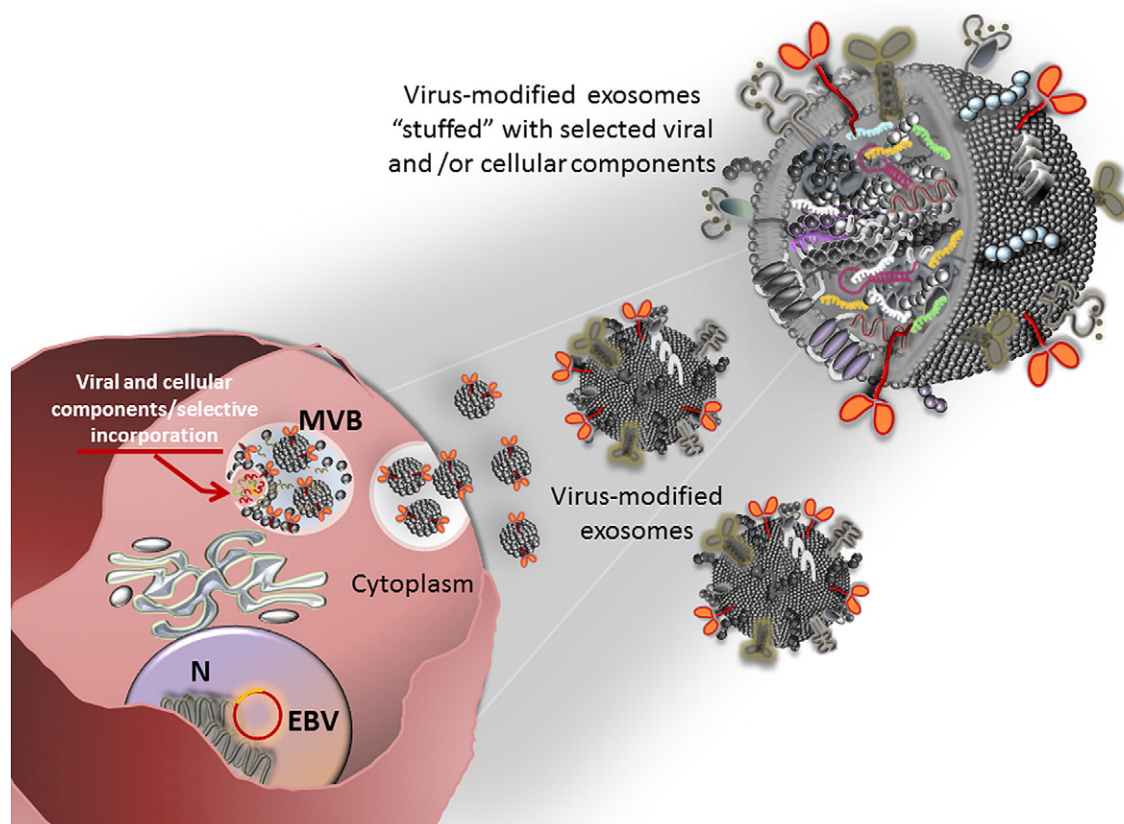


Fig. 1. Formation and release of virus-modified exosomes. Intraluminal vesicles the presumed precursors of exosomes, are formed by inward budding in the lumen of multivesicular bodies (MVBs). The process of internal budding leads to an encapsulation of cytoplasmic content from the cell of origin, including host and/or viral components such as nucleic acids (e.g. small non-coding RNA and mRNA) and proteins (e.g. proteins situated in the cytoplasm, membrane bound proteins, viral (glyco)proteins). The exact mechanism of how nucleic acids are packaged into exosomes is unclear and maybe due to simple (random) inclusion of molecules or by active and selective packaging of certain nucleic acid species such as miRNAs. Induced exosome release and selective cargo incorporation are evident in virus-infected cells. When released to the extracellular environment, exosomes have the same orientation as the cell membrane and have been shown to display many of the surface markers from their cell of origin. While incorporation of viral proteins into exosomes appears to be selective; the criterion determining their exosomal faith is yet to be defined. (Nucleus, N; Epstein - Barr virus, EBV).

sorting into lysosomes. ESCRT-III plays a role in the concentration of the cargo and recruits de-ubiquitylating enzymes to remove the ubiquitin that is necessary before the cargo enters the ILVs [6,8,9]. Cells lacking ESCRT proteins produce fewer MVBs and ILVs and fail to deliver ubiquitylated cargo to lysosomes [10]. Although an essential role for ESCRT in ILV formation has been described in yeast, whereas in mammalian cells ILVs can be formed in an ESCRT-independent manner since simultaneous knockdown of all major ESCRT components did not completely inhibit MVB biogenesis nor ILV formation [11]. Furthermore, there is also evidence that exosome secretion is at least in part ESCRT-independent [12,13]. This leads to the hypothesis that higher eukaryotes use the ESCRT system together with ESCRT-independent pathways, which seems to be driven by lipids, to form ILVs [9,14]. Trajkovic et al. demonstrated that lipid driven pathway may select cargo for protein sorting that requires the sphingolipid ceramide. Another lipid that may be crucially involved in ILV formation is the phospholipid lysobisphosphatidic acid (LBPA). Lipid-rich domains bend inward and form a vesicle in this way. LBPA and ceramide both have a cone-shape structure, which is thought to facilitate the inward transformation of the membrane [6]. It seems that lipids are involved in the formation of ILVs out of MVBs, while ESCRTs are mainly involved in cargo sorting. It is currently unknown what the molecular mechanism(s) are behind the fusion of ILV loaded MVBs with the plasma membrane. SNAREs are molecules of large evolutionary conserved family of proteins that are involved in membrane tethering, docking and fusion [15]. Most SNAREs are type II membrane proteins with highly specific docking properties. When a v-SNARE of one membrane interacts with the t-SNARE of the opposite membrane fusion takes place [16]. Rab, small transport step-specific GTP-binding proteins, and other tethering molecules support the fusion of MVB or lysosomes with the plasma membrane that is necessary for exosome release [15,16].

2.1. Properties of exosomes

Exosomes were first described as external membrane vesicles originating from maturing reticulocytes and seen as a method of these cells to remove obsolete material [17–19]. Subsequent electron-microscopy studies showed fusion events between late endosomes and the plasma membrane in multiple hematopoietic cell types suggesting the content of MVBs was released into the extracellular space as exosomes. Due to their origin exosomes are seen as a specialized type of microvesicles not to be confused with “shedding” vesicles that derive from the plasma membrane. Indeed proteomic analysis together with EM studies strongly suggested that ILVs are intracellular precursors of exosomes [20]. A function for exosomes in cell-cell communication was first proposed by Raposo et al. that showed exosomes from B cells can incorporate and transport functional antigen-presenting complexes [21]. We now know that exosomes are released by most if not all cell types, including dendritic cells, mast cells, T cells, B cells, epithelial cells, mesenchymal stem cells, neuronal cells and many tumor cells [22]. Although exosomes are one of several groups of cell-secreted vesicles, they are relatively well-characterized and defined for their biophysical and biochemical properties compared to other secreted membrane vesicles.

Exosomes are typically purified from the supernatant of cultured cells by ultracentrifugation with 50–100 nm diameter size and a density in sucrose of 1.13–1.19 g/ml. Proteomic analysis has demonstrated that exosomes contain defined sets of lipid and proteins and are carriers of nucleic acids, including messenger RNA (mRNA) and non-coding micro RNA (miRNA) [23,24] (see Fig. 2). While most exosomes share a core set of proteins and lipids, their content is to some extent dependent on the cell type that produces them. It has been speculated that exosomes may derive from different MVB subsets producing distinct subtypes of exosomes. In agreement with this notion our own studies indicated

that two exosomal proteins are localized in distinct endosome-like vesicles [25].

Mass spectrometry contributed to the discovery of over 4000 different proteins identified in exosomes [26]. Whether all these proteins contribute to their function remains to be seen, but there seems to be a clear conserved protein repertoire in exosomes across cell-types and species [27]. For example the endosomal proteins such as Alix and TSG101 have been identified in the majority of the exosomes studied for their protein content thus far. In addition, heat shock proteins, which are involved in protein trafficking, are frequently found in exosomes [28]. Exosomes are further enriched in tetraspanins, like CD9, CD63, CD81 and CD82, which are important molecules for protein-protein interactions in cellular membranes. Tetraspanins bind many proteins, including integrins and MHC molecules [3,29–31]. Specific Rab proteins a highly conserved family of small GTPases that functional as molecular switches and coordinate membrane traffic [32] are often observed in exosomes by mass-spectrometry. Exosomes are also rich in annexins, membrane trafficking proteins that are involved in fusion events. Furthermore cytoskeletal proteins like myosine, actin and tubulin are present in exosomes. Finally, metabolic enzymes, antigen presentation molecules, ribosomal proteins and signal transduction molecules are shown to be present in exosomes [26].

Besides selected sets of proteins, exosomes also incorporate (functional) nucleic acids, most notably small RNA molecules [33] presumably, due to a close intracellular interaction with RISC-assembly complexes situated at endo-lysosomal membranes [34]. Of all RNA molecules detected in exosomes, the class of 22nt long, non-coding miRNAs has received attention since the discovery that miRNAs can be functionally transferred to recipient cells [1,24]. MiRNAs regulate gene expression by binding imperfectly to the 3' untranslated region of the target mRNA that results in translational repression of the mRNA into protein [35,36]. This mechanism is somewhat different from siRNA mediated gene repression as siRNAs are perfectly complementary to their target sequence resulting in mRNA degradation [37]. In essence, miRNA regulated repression of genes is reversible while siRNA mediated repression is not. The biogenesis of miRNAs starts in the nucleus with transcription of DNA by RNA polymerase II into primary miRNA (pri-miRNA) [38]. The pri-miRNA must contain hairpin stem loop structures of approximately 33 basepairs, which is critical for DiGeorge Syndrome Critical Region Gene 8 (DGCR8) protein binding. Thereafter pri-miRNA is cleaved into pre-micro RNA by the Drosha-DGCR8 microprocessor complex formed of RNase III enzyme Drosha and the DGCR8 protein. The formed pre-miRNA is exported from the nucleus to the cytoplasm by Exportin-5 in complex with Ran-GTP, where the pre-miRNA is further cleaved into the 22 nucleotide miRNA by the Dicer-TRBP-PACT complex, consisting of the RNase III enzyme Dicer, transactivation responsive RNA-binding protein (TRBP) and p53-associated cellular protein (PACT). The functional strand is then loaded into the RNA-induced silencing complex (RISC) by Argonaute (AGO) proteins, where the miRNA guides RISC to silence target mRNA. The passenger strand, however, is degraded [5,39,40].

2.2. Endosomes/endocytic pathway

The ability of exosomes to incorporate and transfer functional miRNAs in a cell-type specific manner has drawn much attention by gene-therapists as an attractive alternative for delivery of therapeutic genetic material [23]. The mode of entry for engulfing external material is important to which intracellular compartments the material is directed. For many viruses for instance their specific entry route defines its life cycle. For one, it is important for a virus to either avoid direct uptake to lysosomes, a process called endosomal escape. Alternatively choosing the site of replication is equally important while some viruses replicate in the host cell nucleus, others prefer the cytoplasm while others replicate in specific organelles such as late endosomes or MVBs or assemble at the plasma membrane.

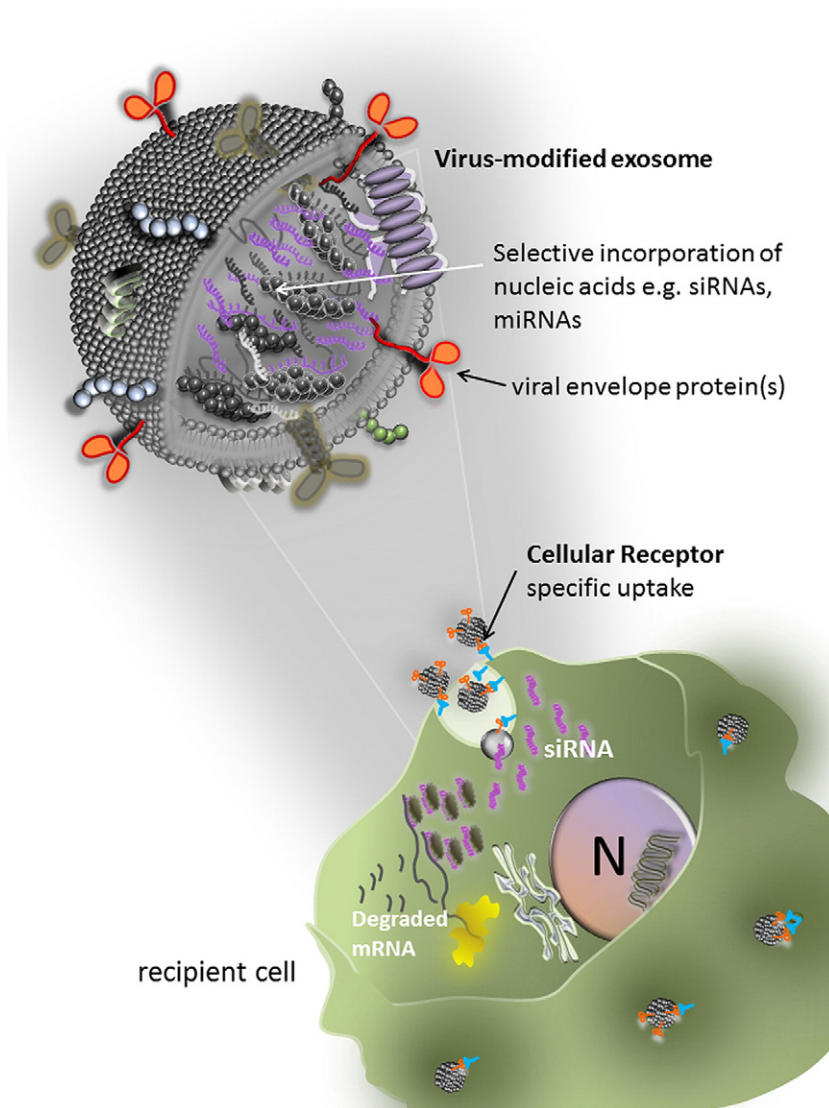


Fig. 2. Extracellular exosomes, expressing a defined set of proteins and lipids, deliver small non-coding RNA to a target cell. When exosomes leave the cell of origin, some will enter the blood stream or other bodily fluids where they can be taken up by other cells as a means of cell-to-cell communication. Depending on a targeting strategy, bioengineered or virus-modified exosomes are destined to engage cell-specific receptors. Exosomes that are taken up by endocytosis will fuse with the endosomal membrane to release their genetic cargo into the cytoplasm where they might associate with the RNAi (RISC) machinery to block mRNA translation into protein. (Nucleus, N).

Little is known how exosomes (or subsets thereof) may enter recipient cells. It has been suggested that this occurs via specific lipid or ligand-receptor interactions. For instance, exosomes that express intercellular adhesion molecule 1 (ICAM1) can bind lymphocyte function-associated antigen 1 (LFA1) expressed by DCs and T cells. Furthermore, exosomes appear to carry phosphatidylserine, which can bind to phosphatidylserine receptors like T cell immunoglobulin domain and mucin domain protein 1 (TIM1) and TIM4 [3]. In addition MHCII positive exosomes could specifically target CD8 + T cells whereas MHC I positive exosomes may target CD4 + T cells. Finally, there is evidence that selected galectins, carried by exosomes from (EBV-infected) B cells can function as specific receptors for TIM(3) molecules on target (T) cells. Despite these points of evidence there is much to be learned about the mechanisms by which (subtypes of) exosomes and their genetic material are transported into recipient cells and whether this transport is functional. Because of the apparent similarities between exosomes and certain RNA viruses, it is useful to briefly revisit how viruses can target their cell of interest and discuss whether targeting of exosomes may function in similar ways.

3. Viral factors in cell targeting and entry

How to enter a cell and to overcome the intracellular barriers for specific delivery of genetic material into defined cellular compartments is perhaps best answered by studying viruses. To multiply, viruses must successfully deliver their genome into host cells while avoiding (innate) immune recognition and exploit the host's intracellular machinery for replication. Efficient viral infection depends on the following sequence of events: (a) binding to cell surface receptors, (b) internalization pathways into cells, (c) escape from endocytic vesicles, and (d) delivery of the viral genome into the nucleus.

To enter the host cell, most viruses first engage with specific cellular membrane proteins or/and receptors that induce conformational changes required for facilitating viral entry. Attachment factors such as proteoglycans or extracellular matrix proteins bind to viruses to concentrate virus particles on the cell surface in a nonspecific way. However, true virus receptors are involved in a more specific binding to target cells. Ultimately these receptors determine which cell types can be infected. A plethora of different surface receptors, entry proteins,

and attachment factors of viruses have been described [41]. As an example, adenoviruses use the coxsackie and adenovirus receptor (CAR) as primary binding receptor and integrins as co-receptors to closely interact with target cells [42]. Other well-known viral entry proteins are for example haemagglutinin, that initiates cell entry for influenza virus and binds with high affinity to the sialic acid receptor [43]. The CD21 complement receptor on B cells is the prime target of the gp350 viral envelope protein of Epstein Barr Virus (EBV), in combination with MHCI that is used as a co-receptor [44]. Thus viruses can interact with their target cells in a very efficient, yet specific manner, ensuring the opening of a defined point of entry that will allow their intracellular voyage towards productive replication. Whether such specificity also exists for cell-secreted exosomes by which they deliver genetic cargo remains to be determined.

Viruses use several distinct endocytic routes for internalization into the host cell: clathrin mediated endocytosis, caveolar endocytosis, and clathrin- and caveolae-independent endocytosis [45]. In addition, some viruses e.g. herpes viruses, retroviruses and paramyxoviruses, seemed to enter cells through direct fusion with the plasma membrane [41,43]. However improvements in rapid visualization techniques revealed that fusion of HIV-1 with the host cell membrane was preceded by receptor-mediated internalization [46]. The simplicity of genome delivery is demonstrated by less sophisticated viruses that use a most efficient method of releasing their genome into the cytosol [47,48]. Non-enveloped adenoviruses, enter cells by classical endocytosis and after internalization, these viruses relocate to endosomes but escape acidification (and degradation in lysosomes) under suitable conditions. The adenovirus disrupts the endosomal membrane by binding of external proteins to the lipid bilayer of the endosome, causing pore formation and releases its capsid into the cytosol [43]. The viral capsid will then dock to the nuclear membrane to deliver viral genome for replication. The lysosomal escape mechanism is well timed, occurring only after a decrease in pH [49]. Interestingly, enveloped viruses including HIV can also fuse with endosomal membranes to mediate viral release into the cytosol thus the ultimately entry site of HIV-1 occurs in the endosomal compartments and not at the plasma membrane. This form of viral fusion to the endosomes is sensitive to a dynamin inhibitor, dynasore, suggesting that the HIV-1 infects cells through a dynamin-dependent fusion event within intracellular (endosomal) compartments [46]. Thus, viruses evolved remarkable strategies to enter the target cells and avoid lysosomal degradation, a dead-end for most exogenous particles that enter via classic endocytic pathways. Insight into the intracellular trafficking of viruses may thus provide a rationale for improving delivery systems of genetic material including synthetic vesicles such as liposomes and naturally occurring exosomes.

3.1. Viral factors can be exploited for targeting therapeutic vesicles

Currently used gene delivery systems can be divided roughly into two groups; the viral and the non-viral vectors. Non-viral vectors, like cationic liposomes, are less toxic and immunogenic than the viral vectors. The cationic liposomes have been improved for a more specific gene transfer. The use of targeting ligands, like transferrin, can improve cellular delivery of cationic liposomes. Furthermore endosomal escape, which is required for the transfer of functional siRNAs or miRNAs is facilitated by lipids such as DOPE [50,51]. However specific organ or tissue targeting of these vesicles remains a big hurdle. In addition, the short lifetime and aggregation of cationic liposomes are problematic for efficient delivery of genetic material that could potentially be overcome by the addition of poly(ethylene glycol) PEG [52], PEG modification could stabilize liposomes and might protect the siRNA from serum degradation, making them more suitable for RNA delivery although this has not yet been clinically validated.

One promising approach is to combine nonviral and viral components, thus taking the advantages of both to efficiently target delivery

of siRNAs. The best known examples are virosomes and virus-like particles (VLPs). Whereas virosomes are vesicles which consist of unilamellar phospholipid bilayer with incorporated virus derived proteins to allow virosomes to fuse with target cells [53], VLPs are made by the transfection of a production cell line with a single plasmid encoding viral structural proteins. Self-assembly of capsid proteins generates non-infectious particles packed with bio-engineered cargo [54]. VLPs are produced by more than 30 different viruses [55] and these VLP have shown to successfully transfer nucleic acids [56–58] However, they exhibit limited packaging capacity and they can be immunogenic [57]. One interesting example of successful -combinatory method is by coating liposomes with inactivated hemagglutinating virus of Japan (HVJ). HVJ liposomes have two important envelope proteins that are involved in membrane fusion and after fusion they can introduce DNA directly into the cytoplasm [59].

4. Viruses manipulate exosome composition and production

Viruses hijack the endosomal pathway or the endosomal machinery for their own benefit, not only to produce new virions in the productive stages, but also for ingenious means of immunoevasion or to facilitate viral spread. Besides by modifying the proteome and genetic content of exosomes, it appears that viruses exploit a whole range of secreted vesicles as recently reviewed by Meckes et al. [60]. HIV for instance exploits the ESCRT machinery, for the formation of a particular exosome-like vesicle that is closely involved with viral budding from the plasma membrane [60–62]. Based on this observation the 'Trojan Horse hypothesis' predicts that some retroviruses maybe packaged specifically within an exosome-like 'coat', that allows a stealthy infection modus of neighboring cells i.e. without the use of envelope glycoproteins (Env) and/or retroviral receptors [63]. In addition, exosomes released from HIV infected cells may contain co-receptors (CCR5) that when transferred to neighboring/recipient cells may enhance their susceptibility to infection by HIV promoting viral spread [64]. Although, many details are unknown, another interesting observation is the specific release and transport of the HIV Nef protein to neighboring cells via exosomes or 'nanotubes' [65]. Nef is able to alter the endosomal system altogether by increasing the number of endosomes, lysosomes and MVBs [64,66]. Nef is widely considered as an HIV virulence factor and one mechanism maybe the secretion via exosomes that is associated with the induction of apoptosis of responding CD4 + T cells [67]. Collectively, these observations link Nef in exosomes to HIV pathogenesis by means of stimulating immuno-evasion.

Our own studies have shown that the EBV encoded latent membrane protein-1, (LMP1) plays an important role in exosome secretion of the infected cells. LMP1 is a potential oncoprotein that induces constitutive NF- κ B activation driving the continuous proliferation of latency type III lymphoblastoid B cell line (LCL) cells in vitro. To avoid over-activation of NF- κ B a condition that is associated with EBV-associated lymphomas and non-EBV associated diffuse large B cell lymphomas [68], LMP1 is not rapidly degraded but instead escapes by secretion via exosomes. This occurs by the selective association of LMP1 with the tetraspanin CD63 that drives sorting and secretion via exosomes. Importantly secreted LMP1 has negative effects on T-cell responses, helping the infected cell to escape from immune responses [25,69] similar to what has been observed for secreted Nef. Interestingly, the EBV-LMP1 protein has some key biochemical characteristics in common with HIV-Nef protein and the gp41 envelope protein. Specifically, LMP1 shares a conserved transmembrane domain with the Env proteins that are carried by proteins of other classes of retroviruses [69].

Since there are many remarkable similarities between viruses and exosomes, it has been suggested that viral immune evasive and targeting properties could be used in artificial non-viral carriers for an optimal target cell selection in drug delivery [70]. Because virus-encoded envelope

proteins exhibit superior binding and entry specificity the efficacy of exosomes delivering genetic material, could be improved by incorporating selected viral proteins [71]. In fact, naturally produced exosomes that are released from EBV producing cells may carry the viral glycoprotein gp350 on their surface [44]. In line with these findings Vallhov et al. suggested that exosomes could be engineered to selectively target CD21 positive B cells, by introducing gp350 in exosomes produced by non-infected cells. Indeed as a proof of principle these authors showed that HEK293 cells overexpressing EBV encoded gp350 release exosomes carrying this protein that specifically target CD21 positive B cells [44]. However caution should be made before administering these exosomes *in vivo* as CD21 is also expressed by other cells such as follicular DCs. Nevertheless, engulfment of gp350-containing exosomes by leukemic blasts (chronic lymphocytic leukemia, B-CLL) and presentation of gp350-derived peptides evoked EBV-specific T cells redirecting strong antiviral cellular response towards malignant B-cells [72]. One could speculate that these 'targeted' exosomes, loaded with an miRNA or siRNA that inhibits the function of an activated oncogene in B-CLL might be very powerful and cell-specific therapeutic tool. It is likely that additional CD21 + cells such as follicular DCs will not be affected by such therapeutic RNA since these cells do not express the target mRNA.

The gB envelope protein of human herpes virus type 1 also gains access to luminal vesicles and is released via exosomes. This observation was interpreted as an immune-evasive function of the virus diverting class II molecules from efficient antigen presentation location within the cell. Yet, the primary function of HSV1-gB is to mediate fusion to the target cell thus permitting viral entry. It thus seems attractive to study gB-driven exosome targeting and uptake by susceptible cells [73]. Moreover, Hepatitis C Virus (HCV) envelop glycoproteins, such as E2, interacts with human tetraspanin CD81 to be sorted into exosomes. These HCV CD81-protein complexes are detected in blood samples of HCV-patients, suggesting yet another role of exosomes in viral pathogenesis [74]. Collectively, these studies indicate that both DNA and retroviruses hijack the endosomal–exosomal pathway not exclusively for their productive life cycle but also for other purposes such as evading immune responses. Alternatively, modified exosomes carrying viral proteins can be used as effective vaccines, indicating the safety of this approach. Besides incorporation of gp350 into naturally produced exosomes similar schemes have been attempted on related carrier vesicles. For instance the incorporation of vesicular stomatitis virus-encoded G protein (VSV-G) into HIV-based viral vectors enhances the uptake of these virus-like particle particles by dendritic cells [75]. Similarly, incorporation of VSV-G coupled to antigen ovalbumin (OVA) into exosome-like vesicles, yields to their elevated uptake by dendritic cells [76]. These DCs that engulfed modified exosomes showed enhanced presentation of exosomal OVA antigens and induced specific CTL responses, suggesting that the targeting of antigens and viral fusion proteins to exosomes improves exosomal vaccines. The efficacy of viral proteins in exosomal vaccines has further been tested in a study from Kuate et al. showing that exosome-based vaccines containing the S protein of the SARS coronavirus induce neutralizing antibody titers [77].

4.1. Virus modified exosomes deliver functional genetic material

Many similarities in biochemical composition, biogenesis and cellular release of exosomes and retroviruses have been mentioned and studied in detail. This has led some to hypothesize that retroviruses may have evolved to utilize a preexisting host endosomal-exosome biogenesis pathway for the formation of infectious virus and their egress [62]. Retroviruses like exosomes contain RNA; whereas the RNA of retroviruses makes up their entire genome, exosomes may contain a diverse population of RNA molecules including miRNA and mRNA [4,63]. HIV-1 assembly appears to take place at the late

endosomal membrane of macrophages, while in T cells, HIV seems to preferentially bud from the plasma membrane. Strikingly, even assembly of HIV at the plasma membrane shares similarities with endosomal budding, for instance the recruitment of ESCRT machinery is required for efficient budding [63]. The fact that exosomes as well as retroviruses require high levels of cholesterol and glycosphingolipids (both contain a common glycan coat with high mannose and complex N-linked glycans) suggest similar requirements for particle assembly. Detection of endosomal markers, such as CD63, in association with macrophage-derived virions again suggests that viral assembly and budding takes a route similar to exosomal release by MVB [78]. Indeed, MVB proteins like E class proteins participate in HIV budding and probably also in other (retro) viruses [79,80].

Baretto et al. showed that infection of epithelial cells with Rotavirus (RV) increases the release of membrane vesicles with typical exosome markers. These exosomes from RV-infected cells showed a higher T-cell inhibition than exosomes from non-infected cells however no viral products were detected in these vesicles [81]. This clearly suggests that the content of exosomes of RV-infected cells is different from the exosomes of non-infected cells. Similar findings in HIV-infected cells indicate specific incorporation and release of the host-encoded anti-viral protein APOBEC3G which is abundantly secreted via exosomes [4,82]. In addition, we have shown that EBV-infected B cells secrete LMP1 via exosomes that have immunomodulatory properties on T cells and DCs [83–85]. Finally, we were first in demonstrating that virus-infected cells package virus-encoded RNAs into exosomes that are delivered into non-infected recipient cells. Specifically, we showed that high copy numbers of EBV encoded BART-miRNAs are transported via exosomes and that these are functional in multiple recipient cells including primary monocyte-derived DCs. Importantly, we also observed transfer of a subset of viral miRNAs from infected to non-infected cells in humans [1] and proposed that exosomes may be designed to deliver small RNA because of their specialized biogenesis and presumed entry route [33]. These observations have later been confirmed by others [60], suggesting that viral factors, once incorporated into the appropriate delivery vesicles such as endogenously produced exosomes, could be exploited for therapeutic RNAi-approaches with increased targeting efficacy.

5. Exosomes current and future perspectives

RNA interference (RNAi) is a gene silencing process in cells that is initiated by small RNA like siRNA, piRNA and miRNAs. These non-coding inhibitory small RNAs are believed to have originated as an innate defense mechanism against viral infections. But soon it was discovered that introduction of these small RNAs into mammalian cells lead to specific inhibition of gene expression. This raised the possibility that RNAi could be exploited *in vivo* for the targeting of disease-causing genes [86–88]. However, siRNAs are hydrophilic and cannot cross membranes on their own. Thus 'naked' siRNAs cannot reach target tissues *in vivo*. Moreover, siRNAs have a very short half-life in the circulation due to the presence of RNase activity in blood. However chemically synthesized siRNAs and vector-encoded siRNAs may have improved molecular integrity *in vivo*, resulting in more stable delivery [88]. Indeed Bitko et al. showed that pulmonary infection in mice caused by respiratory syncytial virus (RSV) and parainfluenza virus (PIV) can be prevented with intranasal siRNAs [89]. Furthermore RNAi can inhibit neuropathic pain in a rat disease model by injecting siRNAs intrathecally [90] and can inhibit viral replication of many viruses *in vitro* and inhibits the survival and growth of tumor cells [91]. Resistance against RNAi in fighting viral infection has been documented indicating that a combinatorial approach against multiple conserved sequences is likely required to prevent the emergence of RNAi-resistant escape viruses [92].

Although siRNAs are potentially very useful and potent therapeutics against many diseases, delivery needs to be optimized [93,94]. Optimal

delivery of these small, hydrophilic siRNAs still remains a major challenge. The current focus is to prevent premature degradation of the siRNAs by serum nucleases [95] and to improve specific cell targeting and to maximize subsequent RNAi loading into the RISC complexes of the target cells [86]. Antibody molecules can be used to deliver siRNAs to specific target cells via cell-surface receptors [96]. Ligand-associated siRNAs also recognize specific antigens on the target cells and are taken up upon binding. Moreover several delivery vesicles like nanoparticles, viruses and liposomes are used to prevent degradation of siRNAs by serum nucleases. Attenuated viral vectors are also frequently used for siRNA delivery, because they provide stable source of nucleic acids for RNAi machinery. However, current siRNA carriers can be cleared by preexisting antibodies, opsonins, complement or coagulation factors [97]. Furthermore the viral vectors can insert viral DNA into the chromosomal DNA, causing genetic dysregulation [98]. Therefore artificial non-viral vesicles are preferred by some such as like lipid nanoparticles that appear very efficient in siRNA delivery. However, liposomes and lipid nanoparticles are also sensitive to be phagocytosed after binding with opsonins or complement factors [99]. Moreover siRNAs in lipid nanoparticles cannot easily escape from the endosome, which is necessary for loading of the siRNAs in the cytoplasmic RISC complexes [86,98]. Polymeric nanoparticles can escape from endosomes but interact with serum proteins that can increase clearance. Furthermore these particles cause 'rupture' of endosomes that enhances endosomal escape but also leads to the co-release of cathepsin B, a strong trigger for undesired inflammation [98]. Polymeric nanoparticles may also accumulate in unintended tissues thereby limiting their effect on the target tissue of interest [98]. Because of these limitations, alternative more efficient approaches are still needed and under development. An attractive alternative approach for safe and efficient siRNA delivery in vivo even to tissues such as the brain is the use of endogenous cell-produced exosomes as (non-viral) delivery vesicles [24,100]. A major advantage of naturally produced exosomes over lipid (nano) particles or viral-vectors is that they apparently can carry functional siRNAs and/or miRNAs across the blood-brain barrier [100].

RNA interference approaches to treat disease have gained much momentum in the recent years and have the potential to treat rare diseases caused by single genes in a cost effective manner. The major challenge is now to deliver the siRNA or miRNAs effectively to the target tissues. We propose here that basic knowledge of exosome biogenesis in combination with detailed understanding of viral delivery strategies may one day overcome this challenge illustrated by the elegant studies [100].

By discussing the current knowledge of naturally occurring RNA containing vesicles, known as exosomes and the viruses that seem to exploit them we hope to raise interest in the use of these vesicles for therapeutic RNAi delivery in the future. Still much is to be learned about how exosomes operate in vivo, their method of action, specific targeting and their kinetics. Indeed, large scale exosome production and isolation still remains a challenge and ultracentrifugation is time consuming that may hamper efforts to use exosomes as vaccines. Moreover the composition of exosomes is not fully elucidated especially at the RNA level and thus artificial production of these naturally occurring vesicles with complex composition may be more difficult than originally perceived. However, if one considers that the HIV genome is 9.8 kb and packaged within a vesicle similar in size to exosomes, one could potentially pack 500 copies of miRNAs in one single exosome. Balaj et al. estimated that one cell may produce between 10 and 30,000 exosomes a day, [101] one could imagine that production of exosomes carrying sufficient therapeutic RNA molecules is certainly feasible at large scale.

It is becoming apparent that cells do not secrete one but multiple sub-populations of exosomes that probably consist of distinct sets of proteins and lipids. Several important proteins, like tetraspanins, annexins and heat shock proteins are found in all exosomes and may have a 'core' function. However distinct tetraspanins may play an important role in target cells selection [102] but many details remain unclear. Viral proteins maybe used to enhance the uptake and delivery of exosomes by specific

target cells as elegantly shown recently by Alvarez et al. [100]. Importantly, exosomes have already proven their biological relevance as cancer vaccines, whereby dendritic cell-derived exosomes can be used to induce immune responses. This is fully compatible with the idea that exosomes are candidate delivery vehicles for therapeutic RNAs. In addition, virus modified exosomes seem to exhibit potent anti-inflammatory properties, that when fully understood could be exploited clinically. Other intriguing possibilities are that targeted miRNA delivery via exosomes could be exploited for instance by overcoming drug resistance. For example, the overproduction of ABC transporters has been documented to cause drug resistance in hepatocellular carcinoma (HCC) [103]. The resistance in HCC due to upregulation of ABC transporter expression is mediated by the downregulation of several tumor suppressor miRNAs. Reintroduction of these miRNAs in the cancer cells may restore the drug sensitivity of these cells. Since there are several viruses that specifically target liver cells, it is not a stretch to engineer exosomes carrying a virus-derived targeting molecule specific for liver (cancer) epithelial cells carrying therapeutic miRNAs as cargo. In conclusion, these tiny vesicles may have a big future in drug delivery.

Acknowledgments

This project is supported by research grants from the Netherlands Organization for Scientific Research (NWO-Veni) to D.M.P. and the Association for International Cancer Research (AICR 11–0157, UK) to D.K.L.

References

- [1] D.M. Pegtel, K. Cosmopoulos, D.A. Thorley-Lawson, M.A. van Eijndhoven, E.S. Hopmans, J.L. Lindenberg, T.D. de Gruij, T. Wurdinger, J.M. Middeldorp, Functional delivery of viral miRNAs via exosomes, *Proc. Natl. Acad. Sci. U. S. A.* 107 (2010) 6328–6333.
- [2] J. Huotari, A. Helenius, Endosome maturation, *EMBO J.* 30 (2011) 3481–3500.
- [3] C. Thery, M. Ostrowski, E. Segura, Membrane vesicles as conveyors of immune responses, *Nat. Rev. Immunol.* 9 (2009) 581–593.
- [4] N. Izquierdo-Useros, M.C. Puertas, F.E. Borrás, J. Blanco, J. Martínez-Picado, Exosomes and retroviruses: the chicken or the egg? *Cell. Microbiol.* 13 (2011) 10–17.
- [5] D.M. Pegtel, M.D. van de Garde, J.M. Middeldorp, Viral miRNAs exploiting the endosomal-exosomal pathway for intercellular cross-talk and immune evasion, *Biochim. Biophys. Acta* 1809 (2011) 715–721.
- [6] D.J. Katzmann, G. Odorizzi, S.D. Emr, Receptor downregulation and multivesicular-body sorting, *Nat. Rev. Mol. Cell Biol.* 3 (2002) 893–905.
- [7] R.L. Williams, S. Urbe, The emerging shape of the ESCRT machinery, *Nat. Rev. Mol. Cell Biol.* 8 (2007) 355–368.
- [8] J.H. Hurley, ESCRT complexes and the biogenesis of multivesicular bodies, *Curr. Opin. Cell Biol.* 20 (2008) 4–11.
- [9] M. Simons, G. Raposo, Exosome—vesicular carriers for intercellular communication, *Curr. Opin. Cell Biol.* 21 (2009) 575–581.
- [10] M. Marsh, M.G. van, Cell biology. No ESCRTs for exosomes, *Science* 319 (2008) 1191–1192.
- [11] S. Stuffers, W.C. Sem, H. Stenmark, A. Brech, Multivesicular endosome biogenesis in the absence of ESCRTs, *Traffic* 10 (2009) 925–937.
- [12] Y. Fang, N. Wu, X. Gan, W. Yan, J.C. Morrell, S.J. Gould, Higher-order oligomerization targets plasma membrane proteins and HIV gag to exosomes, *PLoS Biol.* 5 (2007) e158.
- [13] K. Trajkovic, C. Hsu, S. Chiantia, L. Rajendran, D. Wenzel, F. Wieland, P. Schwillie, B. Brugger, M. Simons, Ceramide triggers budding of exosome vesicles into multivesicular endosomes, *Science* 319 (2008) 1244–1247.
- [14] N. Kosaka, H. Iguchi, Y. Yoshioka, F. Takeshita, Y. Matsuki, T. Ochiya, Secretory mechanisms and intercellular transfer of microRNAs in living cells, *J. Biol. Chem.* 285 (2010) 17442–17452.
- [15] H.R. Pelham, SNAREs and the specificity of membrane fusion, *Trends Cell Biol.* 11 (2001) 99–101.
- [16] T.H. Sollner, Regulated exocytosis and SNARE function (Review), *Mol. Membr. Biol.* 20 (2003) 209–220.
- [17] R.M. Johnstone, Revisiting the road to the discovery of exosomes, *Blood Cells Mol. Dis.* 34 (2005) 214–219.
- [18] R.M. Johnstone, Exosomes biological significance: a concise review, *Blood Cells Mol. Dis.* 36 (2006) 315–321.
- [19] U.J. Dumaswala, T.J. Greenwalt, Human erythrocytes shed exocytic vesicles in vivo, *Transfusion* 24 (1984) 490–492.
- [20] R. Wubbolts, R.S. Leckie, P.T. Veenhuizen, G. Schwarzmann, W. Mobius, J. Hoenschmeyer, J.W. Slot, H.J. Geuze, W. Stoorvogel, Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation, *J. Biol. Chem.* 278 (2003) 10963–10972.

- [21] G. Raposo, H.W. Nijman, W. Stoorvogel, R. Liejendekker, C.V. Harding, C.J. Melief, H.J. Geuze, B lymphocytes secrete antigen-presenting vesicles, *J. Exp. Med.* 183 (1996) 1161–1172.
- [22] C. Thery, L. Zitvogel, S. Amigorena, Exosomes: composition, biogenesis and function, *Nat. Rev. Immunol.* 2 (2002) 569–579.
- [23] S. Lakhali, M.J. Wood, Exosome nanotechnology: an emerging paradigm shift in drug delivery: exploitation of exosome nanovesicles for systemic in vivo delivery of RNAi heralds new horizons for drug delivery across biological barriers, *Bioessays* 33 (2011) 737–741.
- [24] H. Valadi, K. Ekstrom, A. Bossios, M. Sjostrand, J.J. Lee, J.O. Lotvall, Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, *Nat. Cell Biol.* 9 (2007) 654–659.
- [25] F.J. Verweij, M.A. van Eijndhoven, E.S. Hopmans, T. Vendrig, T. Wurdinger, E. Cahir-McFarland, E. Kieff, D. Geerts, R. van der Kant, J. Neeffjes, J.M. Middeldorp, D.M. Pegtel, LMP1 association with CD63 in endosomes and secretion via exosomes limits constitutive NF-kappaB activation, *EMBO J.* 30 (2011) 2115–2129.
- [26] S. Mathivanan, H. Ji, R.J. Simpson, Exosomes: extracellular organelles important in intercellular communication, *J. Proteomics* 73 (2010) 1907–1920.
- [27] R.J. Simpson, S.S. Jensen, J.W. Lim, Proteomic profiling of exosomes: current perspectives, *Proteomics* 8 (2008) 4083–4099.
- [28] S.M. van Dommelen, P. Vader, S. Lakhali, W.W. van Solinge, M.J. Wood, R.M. Schiffelers, Microvesicles and exosomes: opportunities for cell-derived membrane vesicles in drug delivery, *J. Control. Release* 161 (2011) 635–644.
- [29] J.M. Escola, M.J. Kleijmeer, W. Stoorvogel, J.M. Griffith, O. Yoshie, H.J. Geuze, Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes, *J. Biol. Chem.* 273 (1998) 20121–20127.
- [30] S. Keller, M.P. Sanderson, A. Stoek, P. Altevogt, Exosomes: from biogenesis and secretion to biological function, *Immunol. Lett.* 107 (2006) 102–108.
- [31] W. Stoorvogel, M.J. Kleijmeer, H.J. Geuze, G. Raposo, The biogenesis and functions of exosomes, *Traffic* 3 (2002) 321–330.
- [32] H. Stenmark, Rab GTPases as coordinators of vesicle traffic, *Nat. Rev. Mol. Cell Biol.* 10 (2009) 513–525.
- [33] A. Zomer, T. Vendrig, E.S. Hopmans, E.M. van, J.M. Middeldorp, D.M. Pegtel, Exosomes: fit to deliver small RNA, *Commun. Integr. Biol.* 3 (2010) 447–450.
- [34] D. Gibbins, O. Voinnet, Control of RNA silencing and localization by endolysosomes, *Trends Cell Biol.* 20 (2010) 491–501.
- [35] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, *Cell* 116 (2004) 281–297.
- [36] J. Brennecke, A. Stark, R.B. Russell, S.M. Cohen, Principles of microRNA-target recognition, *PLoS Biol.* 3 (2005) e85.
- [37] A.L. Jackson, P.S. Linsley, Noise amidst the silence: off-target effects of siRNAs? *Trends Genet.* 20 (2004) 521–524.
- [38] S. Sassen, E.A. Miska, C. Caldas, MicroRNA: implications for cancer, *Virchows Arch.* 452 (2008) 1–10.
- [39] J. Winter, S. Jung, S. Keller, R.I. Gregory, S. Diederichs, Many roads to maturity: microRNA biogenesis pathways and their regulation, *Nat. Cell Biol.* 11 (2009) 228–234.
- [40] J. Krol, I. Loedige, W. Filipowicz, The widespread regulation of microRNA biogenesis, function and decay, *Nat. Rev. Genet.* 11 (2010) 597–610.
- [41] M. Marsh, A. Helenius, Virus entry: open sesame, *Cell* 124 (2006) 729–740.
- [42] G.R. Nemerow, P.L. Stewart, Role of alpha(v) integrins in adenovirus cell entry and gene delivery, *Microbiol. Mol. Biol. Rev.* 63 (1999) 725–734.
- [43] D.S. Dimitrov, Virus entry: molecular mechanisms and biomedical applications, *Nat. Rev. Microbiol.* 2 (2004) 109–122.
- [44] H. Vallhov, C. Gutzeit, S.M. Johansson, N. Nagy, M. Paul, Q. Li, S. Friend, T.C. George, E. Klein, A. Scheynius, S. Gabrielsson, Exosomes containing glycoprotein 350 released by EBV-transformed B cells selectively target B cells through CD21 and block EBV infection in vitro, *J. Immunol.* 186 (2011) 73–82.
- [45] S.B. Sieczkarski, G.R. Whittaker, Dissecting virus entry via endocytosis, *J. Gen. Virol.* 83 (2002) 1535–1545.
- [46] K. Miyachi, Y. Kim, O. Latinovic, V. Morozov, G.B. Melikyan, HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes, *Cell* 137 (2009) 433–444.
- [47] M. Kielian, F.A. Rey, Virus membrane-fusion proteins: more than one way to make a hairpin, *Nat. Rev. Microbiol.* 4 (2006) 67–76.
- [48] M. Kielian, Class II virus membrane fusion proteins, *Virology* 344 (2006) 38–47.
- [49] Y.W. Cho, J.D. Kim, K. Park, Polycation gene delivery systems: escape from endosomes to cytosol, *J. Pharm. Pharmacol.* 55 (2003) 721–734.
- [50] M.D. Brown, A.G. Schatzlein, I.F. Uchegbu, Gene delivery with synthetic (non viral) carriers, *Int. J. Pharm.* 229 (2001) 1–21.
- [51] Y. Zhang, H. Li, J. Sun, J. Gao, W. Liu, B. Li, Y. Guo, J. Chen, DC-Chol/DOPE cationic liposomes: a comparative study of the influence factors on plasmid pDNA and siRNA gene delivery, *Int. J. Pharm.* 390 (2010) 198–207.
- [52] C.R. Dass, P.F. Choong, Selective gene delivery for cancer therapy using cationic liposomes: in vivo proof of applicability, *J. Control. Release* 113 (2006) 155–163.
- [53] A. Huckriede, L. Bungener, T. Stegmann, T. Daemen, J. Medema, A.M. Palache, J. Wilschut, The virosome concept for influenza vaccines, *Vaccine* 23 (Suppl. 1) (2005) S26–S38.
- [54] Y. Seow, M.J. Wood, Biological gene delivery vehicles: beyond viral vectors, *Mol. Ther.* 17 (2009) 767–777.
- [55] R. Noad, P. Roy, Virus-like particles as immunogens, *Trends Microbiol.* 11 (2003) 438–444.
- [56] B.C. Bundy, M.J. Franciszkowicz, J.R. Swartz, *Escherichia coli*-based cell-free synthesis of virus-like particles, *Biotechnol. Bioeng.* 100 (2008) 28–37.
- [57] A. Touze, L. Bousarghin, C. Ster, A.L. Combata, P. Roingard, P. Coursaget, Gene transfer using human polyomavirus BK virus-like particles expressed in insect cells, *J. Gen. Virol.* 82 (2001) 3005–3009.
- [58] T. Yamada, Y. Iwasaki, H. Tada, H. Iwabuki, M.K. Chuah, T. VandenDriessche, H. Fukuda, A. Kondo, M. Ueda, M. Seno, K. Tanizawa, S. Kuroda, Nanoparticles for the delivery of genes and drugs to human hepatocytes, *Nat. Biotechnol.* 21 (2003) 885–890.
- [59] Y. Kaneda, HVJ liposomes and HVJ envelope vectors, *Cold Spring Harb. Protoc.* 2011 (2011) 1281–1289.
- [60] D.G. Meckes Jr., N. Raab-Traub, Microvesicles and viral infection, *J. Virol.* 85 (2011) 12844–12854.
- [61] P. Benaroch, E. Billard, R. Gaudin, M. Schindler, M. Jouve, HIV-1 assembly in macrophages, *Retrovirology* 7 (2010) 29.
- [62] A. Pelchen-Matthews, G. Raposo, M. Marsh, Endosomes, exosomes and Trojan viruses, *Trends Microbiol.* 12 (2004) 310–316.
- [63] S.J. Gould, A.M. Booth, J.E. Hildreth, The Trojan exosome hypothesis, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 10592–10597.
- [64] M. Mack, A. Kleinschmidt, H. Bruhl, C. Klier, P.J. Nelson, J. Cihak, J. Plachy, M. Stangassinger, V. Erfle, D. Schlondorff, Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection, *Nat. Med.* 6 (2000) 769–775.
- [65] L.L. daSilva, R. Sougrat, P.V. Burgos, K. Janvier, R. Matterna, J.S. Bonifacio, Human immunodeficiency virus type 1 Nef protein targets CD4 to the multivesicular body pathway, *J. Virol.* 83 (2009) 6578–6590.
- [66] R. Madrid, K. Janvier, D. Hitchin, J. Day, S. Coleman, C. Noviello, J. Bouchet, A. Benmerah, J. Guatelli, S. Benichou, Nef-induced alteration of the early/recycling endosomal compartment correlates with enhancement of HIV-1 infectivity, *J. Biol. Chem.* 280 (2005) 5032–5044.
- [67] T.D. Campbell, M. Khan, M.B. Huang, V.C. Bond, M.D. Powell, HIV-1 Nef protein is secreted into vesicles that can fuse with target cells and virions, *Ethn. Dis.* 18 (2008) S2–S9.
- [68] R.E. Davis, V.N. Ngo, G. Lenz, P. Tolar, R.M. Young, P.B. Romesser, H. Kohlhammer, L. Lamy, H. Zhao, Y. Yang, W. Xu, A.L. Shaffer, G. Wright, W. Xiao, J. Powell, J.K. Jiang, C.J. Thomas, A. Rosenwald, G. Ott, H.K. Muller-Hermelink, R.D. Gascoyne, J.M. Connors, N.A. Johnson, L.M. Rimsza, E. Campo, E.S. Jaffe, W.H. Wilson, J. Delabie, E.B. Smeland, R.I. Fisher, R.M. Brazier, R.R. Tubbs, J.R. Cook, D.D. Weisenburger, W.C. Chan, S.K. Pierce, L.M. Staudt, Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma, *Nature* 463 (2010) 88–92.
- [69] J.M. Middeldorp, D.M. Pegtel, Multiple roles of LMP1 in Epstein-Barr virus induced immune escape, *Semin. Cancer Biol.* 18 (2008) 388–396.
- [70] D. Sun, X. Zhuang, X. Xiang, Y. Liu, S. Zhang, C. Liu, S. Barnes, W. Grizzle, D. Miller, H.G. Zhang, A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes, *Mol. Ther.* 18 (2010) 1606–1614.
- [71] D. Bedi, T. Musacchio, O.A. Fagbohun, J.W. Gillespie, P. Deinnocentes, R.C. Bird, L. Bookbinder, V.P. Torchilin, V.A. Petrenko, Delivery of siRNA into breast cancer cells via phage fusion protein-targeted liposomes, *Nanomedicine* 7 (2011) 315–323.
- [72] R. Ruiss, S. Jochum, R. Mocikat, W. Hammerschmidt, R. Zeidler, EBV-gp350 confers B-cell tropism to tailored exosomes and is a neo-antigen in normal and malignant B cells—a new option for the treatment of B-CLL, *PLoS One* 6 (2011) e25294.
- [73] S. Temme, A.M. Eis-Hubinger, A.D. McLellan, N. Koch, The herpes simplex virus-1 encoded glycoprotein B diverts HLA-DR into the exosome pathway, *J. Immunol.* 184 (2010) 236–243.
- [74] F. Masciopinto, C. Giovani, S. Campagnoli, L. Galli-Stampino, P. Colombatto, M. Brunetto, T.S. Yen, M. Houghton, P. Pileri, S. Abrignani, Association of hepatitis C virus envelope proteins with exosomes, *Eur. J. Immunol.* 34 (2004) 2834–2842.
- [75] F. Buseyne, G.S. Le, C. Boccaccio, J.P. Abastado, J.D. Lifson, L.O. Arthur, Y. Riviere, J.M. Heard, O. Schwartz, MHC-I-restricted presentation of HIV-1 virion antigens without viral replication, *Nat. Med.* 7 (2001) 344–349.
- [76] V.V. Temchura, M. Tenbusch, G. Nchinda, G. Nabi, B. Tippler, M. Zelenyuk, O. Wildner, K. Uberla, S. Kuate, Enhancement of immunostimulatory properties of exosomal vaccines by incorporation of fusion-competent G protein of vesicular stomatitis virus, *Vaccine* 26 (2008) 3662–3672.
- [77] S. Kuate, J. Cinatl, H.W. Doerr, K. Uberla, Exosomal vaccines containing the S protein of the SARS coronavirus induce high levels of neutralizing antibodies, *Virology* 362 (2007) 26–37.
- [78] M. Deneka, A. Pelchen-Matthews, R. Byland, E. Ruiz-Mateos, M. Marsh, In macrophages, HIV-1 assembles into an intracellular plasma membrane domain containing the tetraspanins CD81, CD9, and CD53, *J. Cell Biol.* 177 (2007) 329–341.
- [79] E. Morita, W.I. Sundquist, Retrovirus budding, *Annu. Rev. Cell Dev. Biol.* 20 (2004) 395–425.
- [80] U.K. von Schwedler, M. Stuchell, B. Muller, D.M. Ward, H.Y. Chung, E. Morita, H.E. Wang, T. Davis, G.P. He, D.M. Cimbora, A. Scott, H.G. Krausslich, J. Kaplan, S.G. Morham, W.I. Sundquist, The protein network of HIV budding, *Cell* 114 (2003) 701–713.
- [81] A. Barreto, L.S. Rodriguez, O.L. Rojas, M. Wolf, H.B. Greenberg, M.A. Franco, J. Angel, Membrane vesicles released by intestinal epithelial cells infected with rotavirus inhibit T-cell function, *Viral Immunol.* 23 (2010) 595–608.
- [82] Y.L. Chiu, V.B. Soros, J.F. Kreisberg, K. Stopak, W. Yonemoto, W.C. Greene, Cellular APOBEC3G restricts HIV-1 infection in resting CD4+ T cells, *Nature* 435 (2005) 108–114.
- [83] J. Flanagan, J. Middeldorp, T. Sculley, Localization of the Epstein-Barr virus protein LMP 1 to exosomes, *J. Gen. Virol.* 84 (2003) 1871–1879.

- [84] D.F. Dukers, P. Meij, M.B. Vervoort, W. Vos, R.J. Scheper, C.J. Meijer, E. Bloemena, J.M. Middeldorp, Direct immunosuppressive effects of EBV-encoded latent membrane protein 1, *J. Immunol.* 165 (2000) 663–670.
- [85] C. Keryer-Bibens, C. Pioche-Durieu, C. Villemant, S. Souquere, N. Nishi, M. Hirashima, J. Middeldorp, P. Busson, Exosomes released by EBV-infected nasopharyngeal carcinoma cells convey the viral latent membrane protein 1 and the immunomodulatory protein galectin 9, *BMC Cancer* 6 (2006) 283.
- [86] M. Dominska, D.M. Dykxhoorn, Breaking down the barriers: siRNA delivery and endosome escape, *J. Cell Sci.* 123 (2010) 1183–1189.
- [87] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, *Nature* 411 (2001) 494–498.
- [88] P. Shankar, N. Manjunath, J. Lieberman, The prospect of silencing disease using RNA interference, *JAMA* 293 (2005) 1367–1373.
- [89] V. Bitko, A. Musiyenko, O. Shulyayeva, S. Barik, Inhibition of respiratory viruses by nasally administered siRNA, *Nat. Med.* 11 (2005) 50–55.
- [90] G. Dorn, S. Patel, G. Wotherspoon, M. Hemmings-Mieszczyk, J. Barclay, F.J. Natt, P. Martin, S. Bevan, A. Fox, P. Ganju, W. Wishart, J. Hall, siRNA relieves chronic neuropathic pain, *Nucleic Acids Res.* 32 (2004) e49.
- [91] I. Friedrich, A. Shir, S. Klein, A. Levitzki, RNA molecules as anti-cancer agents, *Semin. Cancer Biol.* 14 (2004) 223–230.
- [92] Y.P. Liu, K.J. von Eije, N.C. Schopman, J.T. Westerink, B.O. ter, J. Haasnoot, B. Berkhout, Combinatorial RNAi against HIV-1 using extended short hairpin RNAs, *Mol. Ther.* 17 (2009) 1712–1723.
- [93] X.F. Qin, D.S. An, I.S. Chen, D. Baltimore, Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 183–188.
- [94] I. Zidi, N. Ben Amor, Nanoparticles targeting HLA-G for gene therapy in cancer, *Med. Oncol.* 29 (2012) 1384–1390.
- [95] D.A. Braasch, S. Jensen, Y. Liu, K. Kaur, K. Arar, M.A. White, D.R. Corey, RNA interference in mammalian cells by chemically-modified RNA, *Biochemistry* 42 (2003) 7967–7975.
- [96] E. Song, P. Zhu, S.K. Lee, D. Chowdhury, S. Kussman, D.M. Dykxhoorn, Y. Feng, D. Palliser, D.B. Weiner, P. Shankar, W.A. Marasco, J. Lieberman, Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors, *Nat. Biotechnol.* 23 (2005) 709–717.
- [97] R. Waehler, S.J. Russell, D.T. Curiel, Engineering targeted viral vectors for gene therapy, *Nat. Rev. Genet.* 8 (2007) 573–587.
- [98] J.G. van den Boorn, M. Schlee, C. Coch, G. Hartmann, SiRNA delivery with exosome nanoparticles, *Nat. Biotechnol.* 29 (2011) 325–326.
- [99] M.A. Dobrovolskaia, P. Aggarwal, J.B. Hall, S.E. McNeil, Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle biodistribution, *Mol. Pharm.* 5 (2008) 487–495.
- [100] L. Alvarez-Erviti, Y. Seow, H. Yin, C. Betts, S. Lakhali, M.J. Wood, Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes, *Nat. Biotechnol.* 29 (2011) 341–345.
- [101] L. Balaj, R. Lessard, L. Dai, Y.J. Cho, S.L. Pomeroy, X.O. Breakefield, J. Skog, Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences, *Nat. Commun.* 2 (2011) 180.
- [102] S. Rana, M. Zoller, Exosome target cell selection and the importance of exosomal tetraspanins: a hypothesis, *Biochem. Soc. Trans.* 39 (2011) 559–562.
- [103] F. Borel, L.R. van, A. Koornneef, P. Maczuga, T. Ritsema, H. Petry, S.J. van Deventer, P.L. Jansen, P. Konstantinova, In vivo knock-down of multidrug resistance transporters ABC1 and ABC2 by AAV-delivered shRNAs and by artificial miRNAs, *J. RNAi Gene Silencing* 7 (2011) 434–442.