SiRNA delivery with exosome nanoparticles

Jasper G van den Boorn, Martin Schlee, Christoph Coch & Gunther Hartmann

A natural system for ferrying RNA between cells is used to transport siRNA to the mouse brain.

The luster of RNA interference (RNAi) has dimmed in recent months as the barriers to clinical translation have become more evident. Among these barriers, one of the most important is delivery—the ability to target RNAi agents, such as small interfering (si)RNA, to specific tissues at therapeutic doses. In this issue, Alvarez-Erviti *et al.*[1] propose to deliver siRNA by harnessing the body's own intercellular shuttle service for RNAs. By expressing a neuron-targeting protein on the surface of exosomes, filling them with siRNA and injecting them into the bloodstream of mice, they achieve specific gene knockdown in the brain (**Fig. 1a**). The use of exosomes as an siRNA carrier, although still in its early days, may be considered a major advance for the field of macromolecular drug delivery and possibly a key step toward clinical application of siRNA.

Research on therapeutic RNAi has focused on three classes of delivery vehicle: viruses, polycationic polyethylenimine (PEI)-based nanoparticles and liposomes. All three have shown some successes, especially in inducing RNAi in the liver. Nonetheless the broad goal of achieving clinically efficient gene silencing in the desired target tissue, while avoiding immune activation and toxicity, is still far out of reach. Viruses can mediate long-term gene silencing by integrating small hairpin RNA expression cassettes into the genome and can be engineered for specific tissue tropism, but they have major drawbacks (**Fig. 1b**). Viruses can be cleared in the bloodstream by preexisting antibodies and can activate complement or coagulation factors[2]. They can also induce neutralizing antibody responses that prevent repeated administration. Dysregulation of gene expression in the target cell by insertional mutagenesis is a major safety issue, and some viruses integrate only in dividing cells.

Synthetic carrier systems, such as PEI nanoparticles, liposomes or lipid nanoparticles, shield siRNA from degradation in the bloodstream and can be functionalized with targeting moieties. However PEI-RNA complexes accumulate in the liver, lung, spleen and kidney, limiting their utility for other tissues. Cytoplasmic delivery of siRNA after endocytosis of the particles is achieved through the 'proton sponge' effect of PEI, which results in endosomal rupture (**Fig. 1c**). From recent work[3], we know that any rupture or leakage of the endosomal or lysosomal membrane will release cathepsin B, leading to inflammasome activation associated with IL-1 induction and apoptosis, which precludes prolonged use of PEI *in vivo*.

Lipids are currently the preferred approach for siRNA delivery, and efficient delivery has been achieved preferentially to the liver. Nonetheless, liver toxicity remains a serious issue. Lipid nanoparticles have also been used to deliver siRNA to different solid tumors in mice. Liposomes or lipid nanoparticles adsorb opsonins that activate complement and coagulation factors and lead to phagocytosis by the mononuclear phagocyte system, owing to their net charge and size[4]. Lipid nanoparticles also depend on endocytosis, and in principle, all material delivered by endocytosis must escape the endosome to enter the cytosol. As with PEI, this process can lead to cell stress, inflammasome activation and apoptosis[3].

Given the limitations of viral and synthetic systems, it seems almost obvious to take a leaf from nature's book and borrow the natural intercellular communication system embodied by exosomes. Exosomes were first described in 1983 in the course of experiments tracking the recycling of transferrin receptor during the maturation of sheep reticulocytes[5]. They are membrane vesicles, 50–100 nm in diameter, and are secreted by most cell types *in vitro*. Exosomes are of endocytic origin, derived from invaginations of the limiting membrane of the multivesicular body and released to the extracellular milieu upon fusion of the multivesicular body with the plasma membrane. Although many questions remain about exosome function, it is known that they affect the function of neighboring cells through intercellular transfer of mRNA, microRNA, receptors and enzymes[6], and that they are involved in the communication of immune responses[7].

Only one previous study has applied exosomes for drug delivery, using them to target a small-molecule, anti-inflammatory drug to immune cells[8]. With their study in this issue, Alvarez-Erviti *et al.*[1] are the first to deliver macromolecular drugs by this method. They prepared exosomes from immature murine dendritic cells that were engineered to express a fusion of the exosomal membrane protein Lamp2b and a tissue-targeting peptide. The siRNA was loaded by electroporation. After a careful characterization of exosomal siRNA delivery *in vitro*, the authors demonstrated *in vivo* that exosomes displaying neuron-specific rabies viral glycoprotein (RVG) injected into the circulation deliver siRNA to neurons, microglia and oligodendrocytes in the mouse brain. To show the therapeutic potential of their approach, they delivered siRNA against BACE1, which mediates the formation of the peptide that forms β-amyloid plaque and is associated with Alzheimer's disease pathogenesis. BACE1 inhibition led to a significant decrease in brain β-amyloid levels of wild-type mice.

A formidable challenge for the treatment of neurological diseases is the blood-brain barrier, which only allows spontaneous diffusion of lipid-soluble molecules <400 Daltons in size. This prevents ~98% of drugs, nucleic acids and exosomes from spontaneously localizing to the brain. RVG targets the alpha-7-subunit of the nicotinic acetylcholine receptor[9], and likely facilitates trancytosis of the exosomes across the blood-brain barrier. Besides their presence on neurons and brain capillaries, acetylcholine receptors are also abundant at neuro-muscular junctions. The authors illustrate targeting specificity by showing that RVG-exosomes do

Jasper G. van den Boorn, Martin Schlee, Christoph Coch and Gunther Hartmann are at the Institute for Clinical Chemistry and Clinical Pharmacology, University Hospital Bonn, Bonn, Germany.
e-mail: gunther.hartmann@uni-bonn.de

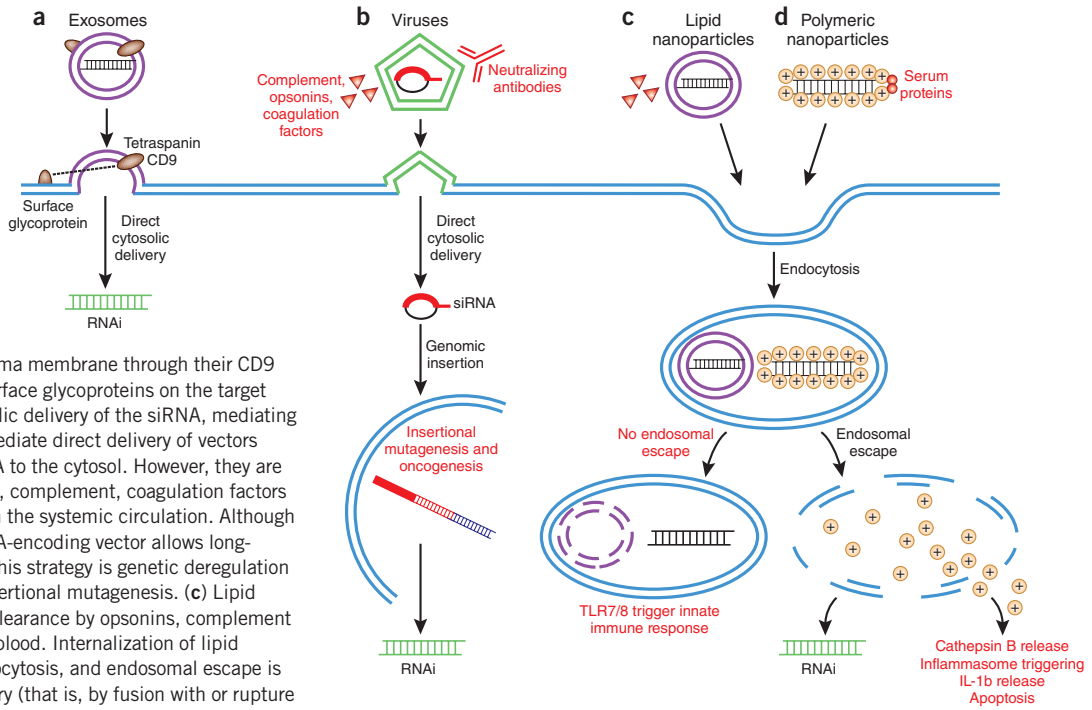

Figure 1 Advantages and drawbacks of siRNA delivery by exosomes, viruses, lipid nanoparticles or polymeric nanoparticles. Major drawbacks are indicated in red, advantages in green. (**a**) Exosomes are not subject to attack by opsonins, complement, coagulation factors or antibodies in the circulation. Dendritic cell-derived exosomes circumvent endocytosis by directly fusing with the plasma membrane through their CD9 tetraspanin interacting with surface glycoproteins on the target cell. This ensures direct cytosolic delivery of the siRNA, mediating temporary RNAi. (**b**) Viruses mediate direct delivery of vectors encoding short hairpin (sh)RNA to the cytosol. However, they are prone to clearance by opsonins, complement, coagulation factors and virus-specific antibodies in the systemic circulation. Although genomic insertion of the shRNA-encoding vector allows long-term RNAi, a potential risk of this strategy is genetic deregulation and oncogene activation by insertional mutagenesis. (**c**) Lipid nanoparticles are sensitive to clearance by opsonins, complement and coagulation factors in the blood. Internalization of lipid nanoparticles depends on endocytosis, and endosomal escape is required for cytoplasmic delivery (that is, by fusion with or rupture of endosomal membrane using viral or bacterial proteins). The presence of RNA in the endosome can lead to unwanted TLR7/8 activation. (**d**) Polymeric nanoparticles such as PEI-siRNA interact with serum proteins, leading to rapid systemic clearance. PEI-siRNA internalization depends on endocytosis, and the complex effectively escapes the endosome by a proton-sponge effect that ruptures the endosome. Endosomal escape of PEI-siRNA or lipid nanoparticles leads to cytosolic delivery of the siRNA, mediating temporary RNAi. However, rupture or leakage of the endosomal/lysosomal membrane in most cases will be associated with cathepsin B release and NLRP3 inflammasome activation. This may result in IL-1 release and apoptosis, complicating prolonged use of this delivery method *in vivo*.

not significantly affect cultured murine C2C12 muscle cells and do target cultured murine Neuro2 neurons *in vitro* and the brain *in vivo*. Additionally, it would have been interesting to determine whether RVG-exosomes target the neuro-muscular interface *in vivo*.

The authors also use a muscle-specific peptide expressed on exosomes to attempt *in vivo* delivery to muscle. The failure of this experiment is unexpected as the peptide has shown *in vivo* efficacy when fused to a cell-penetrating peptide and an antisense-oligo[10]. This result indicates that targeting molecules cannot necessarily be exchanged between delivery approaches, and that selective targeting of siRNA-carrying exosomes to tissues other than the brain may be challenging.

By all appearances, exosomes have multiple advantages over existing siRNA delivery vehicles. Because they could be derived from a patient's own cells, they should be less immunogenic than any foreign delivery vehicle. Exosomes are relatively stable in the blood as they avoid opsonins, coagulation factors and complement, most likely owing to their surface-expression of CD55 and CD59 (ref. 11), as well as antibody responses, owing to their self-derived nature. Dendritic cell–derived exosomes express the tetraspanin CD9 on their surface[12], which facilitates direct membrane fusion with the target cell and contents-delivery directly into the cytosol. This mode of entry bypasses the endosomal-lysosomal pathway, where immunorecognition of nucleic acids by TLR7 and TLR8 occurs. It also circumvents the need for endosomal-escape strategies, with their associated toxicities. Finally, the small size of exosomes should also be beneficial, allowing the particles to avoid phagocytosis by the mononuclear phagocyte system (which clears particles >100 nm in size) and facilitating their extravasation through vessel fenestrations and passage through the extracellular matrix.

Although Alvarez-Erviti *et al.*[1] carried out preliminary research into the immunogenicity of exosomes, much additional work is needed. The authors confirmed that exosomes targeted to the brain by the RVG do not induce short-term innate immune activation *in vivo*, such as induction of IL-6, IP-10, TNF-α or IFN-α. They also found no increase in T-cell proliferation within 3 days in an *in vitro* mixed lymphocyte assay using cells from syngeneic or allogeneic mice. But they did not study whether the RVG–Lamp2b fusion-protein induces adaptive immunity after repeated administration. In fact, adaptive immune responses would be expected. Another issue unaddressed by the authors is that the receptor used by RVG to target the exosomes to the brain, the alpha-7-subunit acetylcholine receptor, is markedly decreased in brains affected by Alzheimer's disease[13]. This issue is of chief importance when aiming to develop RVG-exosomes containing anti-BACE1 siRNA for clinical use in Alzheimer's disease. Finally, it is worth considering that treating patients for extended time periods would require large quantities of autologous monocytes that must be obtained by leukapheresis to generate the required numbers of dendritic cells. These and other questions will surely be addressed in future research.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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