Chapter 7

Summary and Future Perspectives

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Summary

Cell-cell communication was once thought to be operated via secreted factors and direct cell-cell contacts\(^1\). In the late 1960s a salient line of research led to the discovery of bodies in the body fluids that surround the cells in mammalian tissues \(^2\), \(^3\). These bodies were originally insinuated to function as ‘garbage bags’, which cells used to dispose of the unnecessary components building up in the cells\(^4\). The idea that these “vesicle-like” entities could function as cell-cell communicators took shape when it was observed that they seemed to influence the fate of cells, especially in immune responses and cancers\(^5\), \(^6\). This hypothesis was further substantiated by the observation that these vesicles carried miRNA and mRNA which could impart a functional effect in the recipient cells\(^7\). All such lipid vesicles were named as extracellular vesicles (EVs) in 2011\(^8\). Multiple reports in the last decade have testified the important role of EVs in pathological and physiological processes\(^9\)–\(^14\).

EVs are classified into various types (exosomes, microvesicles, apoptotic bodies) based upon their biogenesis pathways\(^15\). Exosomes, the most studied EV type to-date, are formed through the inward budding of the limiting membrane of late endosomes, generating intraluminal vesicles (ILVs), filled with cytoplasmic components. When the thus formed multivesicular bodies (MVBs) fuse with the plasma membrane, the ILVs are released in the extracellular space and now called exosomes. Alternatively, vesicles can form by outward budding of the plasma membrane forming microvesicles, which have been shown to transfer functional biomolecules e.g. proteins and RNA, between cells\(^6\), \(^16\). Apoptotic bodies are vesicles released from dying cells, which have been suggested to serve immune regulatory functions\(^17\), \(^18\). Additional research needs to be done to comprehensively identify and characterize EV types.

Although research on EV functions is rapidly expanding, the mechanism of cargo transfer by EVs to recipient cells is still unclear. A major limitation comes from the fact that the size of EVs (50-200 nm) demands a high-resolution technique for their visualization\(^15\). Moreover, pinpointing EVs at the time that they release their cargo is like finding a needle in a haystack. To address this issue, as described in Chapter 2, investigation of the mechanism of EV cargo release was undertaken using a combinatorial approach involving GFP-loaded EVs, recipient cells expressing cytosolic probes (mAG-galectin3
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and anti-GFP fluobody), and Correlated Light and Electron Microscopy (CLEM)\textsuperscript{19}. Fluorescence light microscopy is a very powerful technique enabling identification of macromolecular interactions within cells using fluorescent probes, including proteins e.g. green fluorescent protein\textsuperscript{20,21}. However, optical imaging precludes the ability to identify individual EVs because the resolution of optical imaging instruments is limited to 200 nm, which means that two adjacent objects can only be resolved if they are located further than 200 nm apart, while EVs themselves are generally smaller than 200 nm (size range 50 nm-200 nm). Electron microscopy (EM) can provide the required high resolution. However fixed samples are used for EM analysis which makes the study of spatiotemporally rare events, like cargo release from EVs, challenging. Therefore, CLEM was employed, which combines Fluorescence and Electron microscopy thereby overcoming the limitations of the separate techniques, thus enabling high-resolution visualization of cellular events\textsuperscript{22,23}.

To identify the intracellular events of cargo release from EVs, EVs were engineered to contain GFP proteins, while recipient cells were engineered to overexpress anti-GFP mCherry antibody fragments (nanobodies) in the cell cytosol. This way, upon incubation of anti-GFP mCherry nanobody-expressing cells with GFP-containing EVs, the formation of mCherry punctae within the cell cytosol revealed the intracellular sites from where cargo release from EVs occurred. Subsequent, electron microscopic investigation revealed the presence of endosomes. Using metabolic inhibitors, it was shown that the process of cargo release is pH- and cholesterol-dependent, with 25% of EV-containing endosomes engaging in cargo release. Altogether, chapter 2 provides interesting insights in the cellular processing and endosomal escape of EVs in recipient cells.

While manipulation of EV cargo can be instrumental in studying EV function, it is also exploited in the development of EVs as drug delivery vehicles. In Chapter 3 we reviewed the hitherto employed strategies used for efficient loading and unloading of EV cargoes, thus giving a comprehensive account of strategies to convert EVs into therapeutic vehicles. It provides an overview of the state-of-the-art methods used for loading and unloading cargoes and discusses advantages and disadvantages of each method for the (un)loading of particular molecular species, including siRNA, miRNA, DNA, and protein. Further, we discuss the upcoming challenges for successful clinical translation and
suggest a workflow that may enable the development of next generation EV therapeutics.

For any given method for EV loading and their downstream application as drug delivery vehicles, it is of crucial importance to characterize their molecular composition comprehensively. Cytosolic overexpression of the cargo is an often-used method to load molecules such as RNA and protein in EVs. However, overexpression of proteins can derail the proper functioning of the producer cells, which may get reflected in the composition of EVs. To investigate this and to test EVs for their therapeutic potential, we engineered EVs for exogenous supplementation of cells with the chaperone DNAJB6, which has been shown to be a powerful modulator of polyQ aggregation in cellular and mouse Huntington’s disease (HD) models. Towards realizing the therapeutic potential of DNAJB6-EVs, it is important to characterize the molecular profiles of these EVs in order to identify possible safety hazards. Chapter 4 describes a comprehensive proteomic analysis of EVs isolated from wild type, DNAJB6-overexpression, and mutant DNAJB6-overexpression HEK293T cell lines. EVs from the overexpression cell lines contained a higher amount of wild type and mutant DNAJB6, respectively, along with other chaperones. Further, it was observed that no other particular class of proteins was enriched in EVs from the overexpression cell lines suggesting a functional state comparable to wild type EVs. Taken together, this study suggests that EVs loaded with the chaperone DNAJB6 through cytosolic overexpression in donor cells hold potential for drug delivery.

To examine if DNAJB6-EVs bring about the desired phenotypic modulation in recipient cells, in Chapter 5 of this thesis, the delivery potential of neural stem cell derived EVs in cellular and mouse Huntington’s disease (HD) models was assessed. We show that EVs containing a high amount of DNAJB6, loaded either by DNAJB6 overexpression in producer cells or by means of a commercially available exosome protein packaging system called XPack, show significant reduction in polyQ aggregation in a cellular HD model. When administered intrathecally in the R6/2 HD mouse model, the XPack DNAJB6-EVs showed suppression of polyQ aggregation in the striatum, which is the most affected brain area in HD. Collectively, we show that EVs are able to impart a functional effect in vitro and in vivo, and exogenous supplementation with DNAJB6 via EVs may be an effective strategy for the treatment of HD.
A major bottleneck in therapeutic delivery to the brain is the presence of the blood-brain barrier (BBB), which largely prevents the transport of therapeutics from blood to brain. In chapter 5 intrathecal administration of EVs was performed to circumvent the BBB. Indeed, the majority of studies depicting the therapeutic role of EVs in the brain rely on direct administration methods, mainly intracerebral and intracerebroventricular injection. However, a non-invasive approach is preferred for clinical translation of EVs. Chapter 6 describes the evaluation of the transport potential of neural stem cell-derived EVs across an in vitro BBB. As a proof-of-concept, EVs were loaded with a protein cargo (mCherry) using the XPack packaging system to enable tracking by fluorescence imaging as well as western blotting. Incubating EVs at the apical (blood) side of an established BBB model consisting of hCMEC/D3 brain endothelial cells, resulted in EV uptake via dynamin-dependent endocytosis following interaction with heparan sulfate proteoglycans (HSPGs). Using fluorometric and immunoblotting analysis, we showed that part of the endocytosed EVs reached the basal side of the BBB, indicating a transcytotic capacity. Collectively, the research described in this chapter suggests that EVs may act as efficient carriers for drug delivery across the BBB. In addition, the data suggest that targeting of nanotherapeutics to HSPG cell surface receptors may improve their binding and/or transport across the BBB.

Overall, the work described in this thesis has provided important insights into EV biology and their potential as biomolecule delivery vehicles.

Future perspectives

Since the discovery of exosomes, continuous research has shaped and reshaped our understanding about extracellular vesicles and their role as cellular messengers. The study of their fundamental biology and engineering remains an active area of research. Several clinical trials have been undertaken to test their potential as delivery vehicles. For example, autologous tumor cell-EVs were used to deliver chemotherapeutics in lung cancer patients, which showed a beneficial clinical response. Similarly, autologous dendritic cell-derived EVs were used to vaccinate patients with metastatic melanoma, resulting in antibody production against melanoma in some of the patients. Although these studies highlighted EVs as feasible and safe biological medicine, the EVs were purified with disparate isolation techniques, and analyzed with different potency assays.
and diverse quality assessment criteria. Therefore, standardized methods for EV production, quality control, and functional analysis are needed in order to compare the outcomes of different studies. The lack of such standardized procedures puts a limit on the scale up of EV production and loading with the desired therapeutics, decelerating their clinical translation. Moreover, parameters for their optimal storage, bioactivity, and stability are not well-defined giving rise to a large variance in used methodologies, which makes the comparison and interpretation of results that are generated in different studies and laboratories difficult. Systematic optimization of protocols will be pivotal in achieving successful clinical application of EVs. Lastly, as most EV effects have been based on in vitro results which do not always translate into efficient therapeutic potential in vivo, and do not always represent physiological conditions, developing relevant (genetically engineered) animal models which can carefully depict the EV efficacy can greatly help the further understanding and development of EV biology and therapy.

Taken together, we seem tantalizingly close to see EVs, nature’s own delivery tools, become a universal platform for potent multifunctional nanomedicine.
References


and Release of Their Cargo from Endosomes. 


