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Tumor exosomes: from loading to release

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CHAPTER 8 | **SUMMARY & GENERAL DISCUSSION**

Summary & Discussion

In this thesis we explored important fundamental and physiological aspects of tumor exosomes. In the first chapters we studied intracellular oncoprotein trafficking, turnover, sorting, and their effects on downstream oncogenic signaling. In the later chapters we used live imaging techniques *in vitro* and *in vivo* to explore molecular mechanisms and physiological properties of tumor exosome release and their role in tumor progression. As a starting point we began our studies on specialized (TSPAN) proteins that form membrane micro-domains and acquire signaling complexes at the limiting membrane of endosomal multivesicular bodies (MVB) to activate signaling pathways. In addition, we attempted to unravel which proteins may be involved in MVB-PM fusion and to demonstrate the relevance of exosome secretion for cancer cells using functional *in vitro* assays and mouse models.

We exploited the unique features of EBV-encoded oncoprotein LMP1 to explore determinants for late-endosomal targeting, namely its association with TSPAN CD63 and the role of palmitoylation as post-translational modification, and investigated the effect of secretion via exosomes on intracellular signaling. Targeting of LMP1 into the endo-exosomal pathway can be viewed as an alternative to lysosomal degradation, as it attenuated LMP1's capacity to activate the NF- κ B pathway. We developed a CD63 pH-sensitive optical reporter to visualize MVB-PM fusion in cancer cells. We demonstrated that MVB-PM fusion in cancer cells occurs continuously at a low rate compared to "regular" transport vesicles. However, the MVB-PM fusion rate was increased by stimulation with histamine and could be decreased by blocking SNARE-mediated fusion. Finally, in a collaborative effort, we provide *in vivo* evidence that breast tumors in a mouse model transfer functional mRNA locally and systemically via exosomes promoting metastatic behaviour in recipient cells. This chapter will briefly summarize the most important observations in these studies and discuss future perspectives.

We studied the effect of exosomal discharge of a viral oncoprotein from cancer cells on signaling, as an alternative mechanism to lysosomal degradation in regulating oncogenic potential. To this end we studied the viral signaling homologue of human CD40, Latent Membrane Protein 1 (LMP1), encoded by the ubiquitous Epstein-Barr Virus (EBV), that similar to many cellular oncoproteins is constitutively active.

Constitutive activation of NF κ B by this viral oncogene may have an important role in viral persistence, but poses a risk for the development of EBV-associated lymphomas. LMP1 activates NF κ B via binding of TRAFs, but is not controlled by phosphorylation and/or ligand binding. We observed somewhat surprisingly that LMP1 escapes lysosomal degradation and is not rapidly degraded through the proteasome either, as previously has been suggested¹. We investigated the role of the LMP1 lipid-raft anchoring sequence FWLY as well as ubiquitylation of the N-terminus. Neither the FWLY-domain nor forced ubiquitination had an effect on LMP1 sorting into exosomes. Instead, we found that LMP1 associates and traffics with the intracellular TSPAN CD63 into vesicles that seem to maintain low cholesterol levels. Strikingly, unlike CD63 in LMP1^{-ve} cells, these low cholesterol levels were maintained even under chemically

induced 'cholesterol-trapping' conditions. Because a large pool of LMP1 was rapidly secreted via exosomes^{2,3}, at least within 24 hours of production, we speculated that LMP1 was in fact a stable protein, in contrast to what was previously believed^{1,4,5}. If exosome release is physiologically relevant, we reasoned that rapid incorporation and secretion of a stable constitutively active signaling protein via exosomes might effect downstream signaling. Next, we investigated the contribution of exosomal discharge of LMP1 to its constitutive activation of NFκB. We found that prevention of LMP1 secretion by knock-down of CD63 dramatically increased LMP1-mediated NFκB signaling. This suggested that exosomal-discharge of (onco)proteins via association with TSPANs is an alternative mechanism to degradation and may be an acquired feature of cancer cells to restrain overstimulation^{6,7}.

We subsequently studied the mechanism of signal termination at late-endosomal membranes and possible molecular determinants of LMP1 exosomal sorting, including the role of palmitoylation in the late-endosomal targeting of LMP1. Endocytosis of activated signaling receptors has classically been viewed as the pathway for attenuation of signal transduction. In recent years, intracellular endosomes were identified as integral platforms for signaling initiation and attenuation^{8,9}. Prolonged signaling of activated receptors from endosomes appears to promote cellular transformation, although the mechanisms remain poorly defined and may be highly context dependent. We demonstrated that LMP1-TRAF2 signaling complexes at endosomal membranes dissociate upon sorting of LMP1 into CD63-enriched exosomes. Provocatively, mutations in two LMP1 C-terminal cytosolic domains, known as transformation effector sites 1 and 2 (TES1 and TES2), promote exosomal sorting of LMP1. TRAF2 (but not TRAF3 and TRAF6) is recruited by LMP1, and TRAF2-LMP1 complexes accumulate at endosomal membranes. Because TRAF2 is not sorted into exosomes we reasoned that active LMP1-TRAF2 signaling complexes dissociate at late-endosomal membranes, facilitating LMP1 sorting and release via exosomes. Alternatively, the endosomal LMP1 is sorted into exosomes by an unknown machinery that forces dissociation with TRAF2. Future studies should decipher whether ESCRT complexes or perhaps TSPAN domain formation and/or inward budding may have a role in this process, for instance through knock-down (KD) studies of specific ESCRT members. Moreover, we showed that the targeting of LMP1 to this defined subcellular location is controlled by a single cysteine residue (C78). Mutational inhibition of C78-palmitoylation perturbed LMP1 trafficking to endosomal membranes, and as a consequence sorting into exosomes was diminished whereas oncogenic NFκB signaling increased (**Figure 1**). We made similar observations for cellular proto-oncogenes, Src-family kinase members Src and Fyn. We demonstrated that constitutively active Src/Fyn non-palmitoylated forms, in contrast to their wild-type counterparts, accumulate at Rab5+ endosomal membranes, increasing their downstream signaling. The palmitoylated counterparts of Src and Fyn on the other hand traffic to Rab7+ late endosomal membranes for signal termination¹⁰. Thus, post-translational palmitoylation is important for regulation of oncoprotein signaling, inducing sorting into the endosomal-exosomal secretory pathway and limiting its availability to interact with essential signaling adapter molecules.

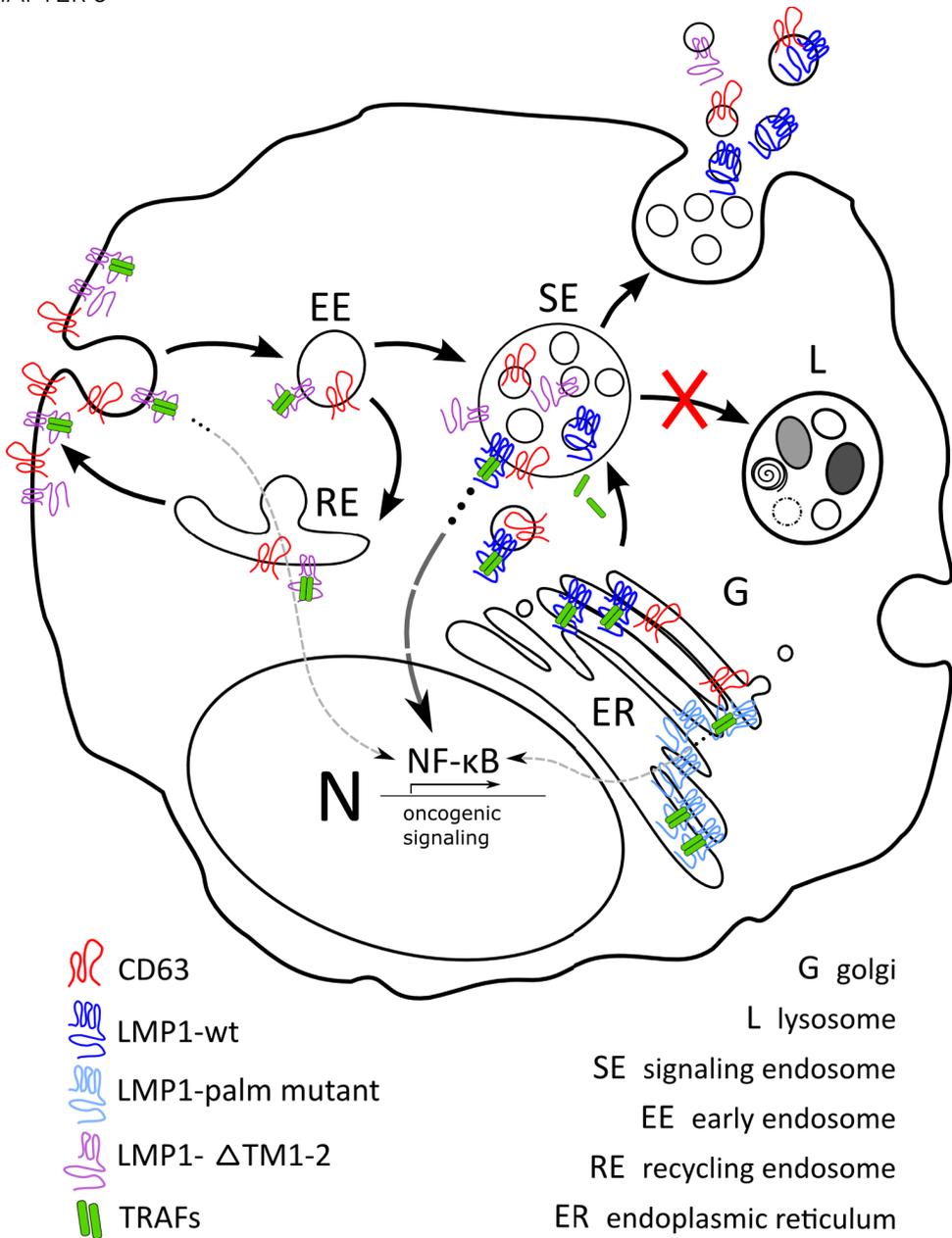


Figure 1 | Model summarising LMP1 sorting into exosomes

Hypothetical model showing that newly synthesized wildtype (wt) LMP1 (dark blue) assembles with CD63 in the ER, traffics to the Golgi (G) where it is palmitoylated and buds off in vesicles from CD63 (red) positive domains that traffic to limiting membranes of signaling endosomes (SE). While palmitoylation targets LMP1 to SE membranes, non-palmitoylated LMP1 (light blue) is retained in the ER/Golgi region. Wild-type LMP1 recruits TRAF2 and the activated signaling complexes accumulate at endosomal membranes activating NFκB. Upon LMP1 sorting into the intra luminal vesicles, LMP1 is then secreted via exosomes while TRAF2 dissociates and is presumably left behind in the cytosol. LMP1 lacking its first trans-membrane (TM) regions (LMP1-ΔTM1-2, purple) accumulates at the plasma membrane (PM) and is endocytosed via a Rab5-Rab7 mediated pathway. The LMP1-palm and the LMP1-ΔTM1-2 mutants are both affected in their NFκB/oncogenic signaling, despite their TRAF2-recruitment capacity seems unaffected.

In a parallel to these studies we developed an imaging tool that directly visualizes MVB fusion with the PM. Exosome-release mechanics and dynamics are an essential, yet unknown part of exosome biology, because tools to follow this process in living cells were either lacking or difficult to quantify. In fact, most *in vivo* and *in vitro* studies that aimed to understand exosome physiology relied thus far on cumbersome isolation techniques. These methods typically involve the collection of large volumes of cultured-cell supernatant over time, and multiple rounds of (ultra) centrifugation, sometimes using density gradients¹¹. A consequence of these isolation approaches is the inevitable loss of the physiological context of the cell and the inability for direct and quantitative measurements of exosome release. Moreover, this technique most likely yields heterogeneous populations of extracellular vesicles (EVs) including plasma membrane-derived vesicles.

The innovative approach we employed has enabled us to discover a fusion-machinery and signaling pathway involved in exosome release from tumor cells. Specifically, we combined a fluorescent reporter for exosome secretion (CD63-pHluorin) with live imaging of cells using TIRF-microscopy. We used this technique to follow MVB-PM fusion dynamics and provided evidence that SNARE molecules and G-protein-coupled receptor signaling are controlling the release of exosomes. Knock-down of SNARE proteins SNAP23 and Syntaxin-4 decreased the number of MVB-PM fusion events, while stimulation with histamine, a soluble GPCR-ligand and component of the tumor microenvironment (TME), significantly increased the number of fusion events. Additionally, we linked the secretion of matrix metalloproteinases (MMPs), proteins that are part of the extra cellular matrix (ECM) remodelling machinery, with the SNAP23-mediated exosomal pathway, as inhibition of SNAP23 function impaired MMP release and limited the invasive behaviour of these tumor cells¹².

Successful treatment of cancer is complicated by the heterogeneous nature of tumors. The tumor microenvironment (TME) and genetic differences between individual tumor cells lead to differences in metastatic behaviour of cells¹³. To study the contribution of exosomal communication to the metastatic behaviour of tumor cells we collaborated in studies that employed intravital imaging of vesicle transfer between tumor cells *in vivo* using the Cre/LoxP recombination reporter system. This system has successfully been applied in mice to identify communication between neuronal cells¹⁴.

In a mouse breast cancer model it was demonstrated that tumor cells transfer mRNAs locally and systemically via a heterogeneous population of EVs. Gene ontology analyses showed that these EVs were enriched for mRNAs involved in migration and metastasis. By exploiting the Cre/LoxP system we could distinguish cells that took up EVs secreted from a specific donor-cell population from those that did not, both locally and systemically. Moreover, we found that EVs released by malignant cells targeted more benign tumor cells, which subsequently displayed enhanced migratory behaviour and metastatic capacity. Our results combined suggest that the specific effects after uptake of these EVs is mediated, at least in part, by the enriched mRNAs specifically involved in migration and metastasis. This 'transfer of malignancy' from a metastatic to

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a more benign tumor cell illustrates that tumor heterogeneity is much more complex than currently anticipated. This will likely influence our ideas on the mechanisms of tumor progression and the design of optimal treatment strategies¹⁵.

Discussion

Signaling & the exosomal pathway

The classic view of terminating downstream signaling by activated receptors at the PM dictates that entering the endosomal system via endocytosis of the membrane-bound receptors terminates signaling. However, endosomal membranes can also function as signaling stations for endocytosed receptors, providing additional control over various aspects of cellular physiology^{16–22}. Within the endosomal pathway, early endosomes are the first sorting stations where decisions are made for processing and routing of newly synthesized endogenous proteins, or whether internalized proteins (cargo) from the PM will be recycled or routed into the late endo-lysosomal pathway for degradation. Deregulation of these processes is strongly associated with cancer^{8,19,23,24}. Thus, knowledge of the underlying molecular mechanisms controlling these processes may help the identification of novel therapeutic targets. However, apart from a probable role for ubiquitination, data on molecular requirements for exosomal sorting remain limited^{19,25}. In chapter 4 we identified palmitoylation as a targeting motif for the viral oncoprotein LMP1. Targeted mutagenesis of the active palmitoylation site C78²⁶, abolished its sorting into exosomes by interfering with the trafficking of LMP1 to (late) endosomes.

The palmitoylation effect

Palmitoylation is a reversible modification controlled by palmitoyltransferases and palmitoyl protein thioesterases²⁷. Strikingly, alterations in aspartate-histidine-histidine-cysteine (DHHC) palmitoyl transferases DHHC expression levels is linked to cancer^{28–33}. At present more than 20 DHHCs are identified, and with the current major efforts to identify DHHC-substrate specificity, specific targeting of palmitoyl transferases or thioesterases using (reversible) inhibitors might lead to novel treatments for cancer and other diseases^{34,35}. For a long time the dynamics of palmitoylation were not clear. Only recently it became clear that (de-) palmitoylation cycles are required for the proper functioning of specific proteins localized at the plasma membrane, since substitution of palmitoylation by an irreversible membrane-anchor showed perturbation of protein localization³⁶. Our study added to this concept by revealing that palmitoylation is required for (late-) endosomal targeting of various proteins. While in our system the signaling potency of viral-LMP1 did not seem to be increased by palmitoylation-dependent targeting to endosomal membranes, oncogenic properties were decreased at this location. Moreover, we showed that palmitoylation could function as a control mechanism next to other mechanisms like phosphorylation or CD63 association, adding further robustness to the control system preventing over-stimulation. The relative contribution of each of these mechanisms is not clear, as we cannot exclude a certain degree of functional redundancy.

LMP1 intracellular sorting and signaling

Regarding the different mechanisms of protein sorting into exosomes currently known in literature, a similar remark could be made. Phenotypically the effect of CD63 KD and the removal of the active palmitoylation site of LMP1 on the sub-cellular localization of LMP1 are quite similar. Yet, mutation of this palmitoylation site (LMP1-C78A) did not affect co-localization with CD63 while LMP1-C78A was clearly excluded from exosomes. This suggests that both mechanisms work in concert in the endo/exosomal-sorting process of LMP1 but at different levels.

Since LMP1-C78A and LMP1 under CD63KD conditions accumulated in the ER/Golgi region, a rather early step in the secretory pathway, it is hard to define the exact role of CD63-association and palmitoylation in exosomal targeting of LMP1. The exosomal loading process of proteins can theoretically be distinguished in clearly distinct steps, namely protein trafficking towards late endosomes, ILV biogenesis and protein loading into ILVs. In this modular view, both CD63 association and palmitoylation of LMP1 would act on the first step, namely protein trafficking towards late endosomes. However, proteins most likely have multiple functions and/or depend on their molecular context for their respective function. Indeed, CD63 recently has also been implicated in the formation of small subset of ILVs, independent of other mechanisms³⁷. Thus, whether palmitoylation and CD63 association besides LMP1 trafficking towards late endosomes are required for later steps of the exosomal loading process of LMP1 remain to be determined.

The exact timing and role of TRAF2 recruitment at the C-terminus of LMP1 likewise remains to be determined. Initial studies indicated that only knock-down of TRAF2, but not TRAF1, -3, -5 or -6 resulted in a decrease in NF κ B signaling and growth-stimulatory signaling³⁸. Our studies confirmed the selective recruitment of TRAF2 by LMP1 by localization studies using confocal analysis. However, the altered localization of LMP1 due to loss of palmitoylation or overexpression of CD63 did not affect recruitment of TRAF2 by LMP1, while LMP1-mediated NF κ B signaling and/or its transformation capacity were clearly affected. This underlines once more the importance of time-related association/dissociation and localization in the functioning of proteins and prompts for a careful interpretation of results with this type of studies.

Exosomes in cancer biology

One interesting unanswered question is whether (cancer) cells 'purposely' activate and use non-classical secretion pathways to terminate signaling instead of just disposing the signaling components through ubiquitination and proteasomal or lysosomal degradation. One explanation is that rapidly proliferating cancer cells have elevated protein synthesis while 'normal' degrading pathways are overwhelmed.

As outlined in the introduction, the physiological relevance of exosome secretion can be looked at from two ways, namely from the target cell perspective as well as the donor cells' perspective. While the former received most attention in literature, in this thesis we also studied the latter perspective. This underappreciated pathway might be especially relevant to cancer, since it is known for decades that classical degradation pathways are impaired in cancer³⁹. MVBs and lysosomes, often even mentioned in one

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breath, both take part of the highly interconnected endosomal system⁴⁰. The aberrant translational control of synthesis in cancer⁴¹ added to impaired protein degradation is therefore likely to impact the dynamics of exosome secretion in tumor cells. As touched upon in chapter 2, exosomal discharge of (onco)proteins can be an alternative way for the cell to counter supra-physiological levels of (signaling) proteins and concomitant overstimulation. Depending on the cancer, this might result in the transfer of a more malignant phenotype to neighboring cells, as can be the case for gliomas where exosomes loaded with EGFRvIII stimulate a pro-metastatic phenotype in recipient cancer cells⁴². This 'secreting cell' perspective could also balance against the implicit portrayal of cancer 'having a mind of its own' as one encounters in current literature, suggested by terminology such as cancer "hijacking" various cellular pathways⁴³⁻⁴⁶. Although various mechanisms have been described of how tumor cells promote their own progression, some of them notably mediated via exosomes^{42,47-49}, the perception that this might be a 'conscious' action of the tumor is true only so far in that it reflects and still operates in the framework of existing biological processes, including natural cell growth, wound healing, angiogenesis and cell recruitment, though failing cellular control-mechanisms. Apart from their effects on potential target cells, focusing on the physiological processes and dynamics underlying the secretion of exosomes might thus provide valuable contribution(s) to our understanding of cancer.

LMP1 in exosomes and immune evasion

Apart from the immediate effects of exosomal discharge of LMP1 we described for the donor cell, i.e. the prevention of overstimulation, exosomal secretion of LMP1 could also play a role in EBV persistence, especially in the context of immune evasion. While EBV is circulating in memory B-cells and remains mostly latent (latency type I), the virus reactivates from time to time as consequence of plasma cell differentiation of its host cell⁵⁰⁻⁵². Reactivation of EBV from submucosal plasma cells leads to epithelial cell infection and shedding of infectious virus in oral secretions, creating newly infected (transformed) B-cells in sub-mucosal lymphoid follicles⁵³. In addition, EBV-carrying memory B-cells recirculate via nasopharyngeal lymph nodes (Waldyer's ring) and temporarily proliferate by re-expressing the default program of gene expression (latency-II), supporting clonal persistence⁵⁴. Both acute B-cell transformation by EBV and endogenous reactivation carry the inherent danger of malignant transformation and lymphoproliferative disease if not controlled properly⁵⁵. In healthy carriers, for both the viral replicative stage as well as the default and transforming latency stages II and III, virus activity is controlled by an abundant HLA-I and -II restricted T-cell response, in particular in virus-resident oral mucosal sites⁵⁶. This T-cell response permanently occupies a large percentage of the host's immune repertoire and is directed against a restricted set of immunodominant latent and lytic viral gene products⁵⁷. Yet EBV somehow escapes complete clearance by the immune system and manages to persist. Indeed, EBV employs different strategies to prevent recognition and elimination by the immune system and is as such in an equilibrium with the immune system, imbalance of which can lead to the development of different tumors,

such as lymphoproliferative disorders (e.g. Pfeiffer's disease), Hodgkin's lymphoma, Burkitt's lymphoma, and nasopharyngeal carcinoma⁵⁸⁻⁶². EBV is known to encode immune evasion strategies at all stages of the viral life cycle^{59,60,63}. An interesting question is if there is a role for exosomes in the immune evasion strategy of the virus. Indeed, previous studies indicated that exosomes from EBV infected cells and recombinant LMP1 could play a role in immune-suppression^{2,3}. In a more recent study we found that exosomes from an LMP1-inducible cell line (BJAB-LMP1) and a constitutive LMP1-expressing cell line (RN) both were 5-fold less immuno-stimulatory to lymphocytes of a mismatched donor in comparison with exosomes from a control (tTA)-inducible cell line (BJAB-tTA) (Verweij, unpublished data). Induced BJAB-LMP1 cells furthermore formed ~50% less conjugates with Jurkat cells compared to BJAB-tTA cells. This could indicate that LMP1 in cells and/or exosomes is able to partly 'shield' EBV+ cells from being recognized by surveilling T-cells, and as such manages to hide from the immune system by a yet unknown mechanism. Our data combined with the notion that a short isolated peptide of the LMP1 protein, the conserved immunosuppressive domain LALLFWL, is able to actively suppress antigen-, mitogen- and co-stimulation (CD3+CD28)-induced T-cell activation as well as the recombinant full molecule^{2,64} suggests that LMP1 may target the immune synapse by inserting itself in the MHC-II/ co-stimulatory molecule domains or the T-cell receptor complex, preventing recognition or physically disturbing downstream T-cell signaling⁵⁹. In addition, virus-modified exosomes may carry and transfer cargo (e.g miRNA) that can manipulate gene expression in (surrounding) recipient T-cells, thus modulating their responsiveness to activating signals⁶⁵.

Exosomes, metastasis and future perspectives

One could wonder whether the properties attributed to exosomes released by tumor cells mimic normal cell-cell communication underlying tissue homeostasis. For instance the induction of vascular leakiness at pre-metastatic sites by melanoma-derived exosomes⁴⁷ might reflect normal physiological processes such as wound healing. In this study, pre-treatment of mice with melanoma-derived exosomes showed a greater metastatic burden in the lungs and a wider tissue distribution of the metastases compared to the control, including bone and brain, and bone marrow-derived cells (BMDC) recruitment to metastatic sites. Tissue repair- and regeneration are thought to involve proliferation of resident cells as well as the selective recruitment of circulating stem and progenitor cell populations⁶⁶. Indeed, metalloproteinase (MMPs) secretion is a potential mechanism by which hMSCs extravasate into injured tissues *in vivo*⁶⁷, while increased MMP expression in cardiac aging is linked to endothelial dysfunction including vascular leakiness⁶⁸. Notably, we and others linked MMP-2 and -9 secretion to the exosomal pathway⁶⁹, although the exact mechanism of secretion remains to be elucidated. Thus, melanoma exosomes could make the lung more vulnerable to metastasis formation directly by making the lung endothelium more permissile to (tumor) cell extravasation, and indirectly by the induced damage, resulting in a recruitment of bone-marrow cells to aid the repair of the lung endothelium but at the same time promoting tumor cell proliferation and/or activation of tissue fibroblasts⁷⁰.

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It has been suggested in the past that metastasis is most likely a very inefficient process, as only ~0.01% of cancer cells injected into the circulation form metastatic foci⁷¹. While this was initially ascribed to low survival rates in the circulation, two other studies disputed this. By injecting a highly metastatic melanoma cell line (B16F10) directly into mice, indeed only a small proportion of cells successfully formed distant lesions in lungs or liver^{72,73}. Strikingly however, the initial step (extravasation) was relatively efficient (e.g. 80% of the cells trapped extravasated in the liver), while the efficiency of the next stages, i.e. growth of solitary cells and continued growth of early micrometastases into macroscopic tumors, was extremely low (0,02%)⁷². The efficiency of these last steps might be highly dependent on the 'compatibility' of the tumor cell and the 'host' tissue into which it extravasated⁷⁴. What is interesting about these prior studies is that by direct injection of the tumor cells into the bloodstream, the "exosomal conditioning" component might in fact be eliminated whereas Peinado et al. showed that exosomal conditioning of pre-metastatic niches in mice greatly influenced the efficiency of the metastasis process⁴⁷. One of the main outstanding questions in this respect is whether the dosage and frequency of exosomes administered to these mice actually reflects the dynamics of exosome secretion by tumors *in vivo*, which would further support the physiological relevance of the effects described. This question ultimately needs to be answered using an *in vivo* model with solid tumors - ideally with modifiable exosome secretion - instead of intravenously injected tumor cells. We believe that the CD63-pHluorin MVB-PM fusion reporter we developed and applied to various cell lines could also be of great benefit in this respect, since it allows for the discovery of exogenous factors modulating exosome secretion.

Another intriguing question in the context of the (in)efficiency of metastasis and role of exosomes is what could be the contribution to successful metastasis of exosomal communication between tumor cells mutually or between tumor cells and surrounding cells. As extravasation is not a unique feature of the tumor cell but rather independent of their metastatic ability⁷⁵, the successful outgrowth of a metastasized cell highly depends on the cross-talk between the extravasated cancer cell ('seed') and the specific organ microenvironment ('soil'), a proposal originally put forward by Stephen Paget in 1989⁷⁶. This suggests that the more heterogeneous a tumor is, the higher the chance of a 'match' between seed and soil. Indeed, while neoplasms are generally heterogeneous⁷⁷, metastases are clonal⁷⁸. Moreover, increasing genetic instability of clones isolated from murine neoplasms is associated with increasing metastatic potential⁷⁹. While genomic instability is one contributor to the heterogeneity observed in tumors, epigenetic change is another. It has been well documented that cell extracts can induce epigenetic changes in co-cultured target cells, and since exosomes carry genetic material they could potentially induce epigenetic changes, contributing to tumor heterogeneity⁸⁰. Indeed, MV mediated mRNA and protein transfer by embryonic stem cells could reprogram hematopoietic progenitor cells⁸¹. Thus, exosomal communication between tumor cells and/or their environment may contribute to the tumor heterogeneity, and hence increase the likeliness of

extravasated cancer cells with potential to form a secondary tumor. Our study (chapter 7) shows that EV-communication between tumor cells is a physiological process and can result in a change in the metastatic behavior of recipient cells, illustrating that tumor heterogeneity is more complex than currently anticipated. Additionally, this may grant tumor cells a certain degree of 'plasticity', a trait currently ascribed and reserved for cancer-stem cells⁸². This heterogeneity and plasticity influences the therapy resistance of the tumor and thus the overall tumor survival probabilities. Altogether this illustrates that EV/exosomal-communication in cancer is a highly important and intriguing field that is worth exploring, not only for a better understanding of cancer but also for improving treatment strategies in the future.

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