Cancer progression and metastasis formation rely on the bi-directional and dynamic interactions between mutated tumor cells and their complex tumorigenic microenvironment. Tumor cells have the capacity to physically and biochemically modify their surrounding extracellular matrix and the phenotype of stromal cells (which include fibroblasts, endothelial cells, mesenchymal stem cells, macrophages and other types of immune cells) for their own benefit. Reciprocally, the neighboring tumor microenvironment reacts and shapes the tumor to sustain its growth, eventually fostering its aggressiveness. This educated tumor ecosystem will support tumor growth at a primary site, during invasion and throughout the metastasis cascade. In addition, tumor-secreted factors modify the host microenvironment in distant organs, thereby promoting the formation of a premetastatic niche (PMN) and favoring the colonization of metastatic cells. Some studies however suggest that the microenvironment, such as the well described tumor-associated fibroblasts and extracellular matrix components, can have adverse behavior on tumor progression and potentially mediate anti-tumorigenic immune surveillance. Better understanding of this constant dialog within the tumor ecosystem is instrumental in developing appropriate and efficient therapeutic strategies.

In addition to direct cellular interaction or action of soluble factors, communication between a tumor cell and its microenvironment can also be mediated by the secretion and uptake of extracellular vesicles (EVs). Since the late 90s, and the functional characterization of a particular subtype of EV, exosomes, interest in EVs as mediator of cell-cell communication has grown exponentially. EVs are secreted lipid bilayered vesicles encapsulating mRNA, miRNA, proteins and, in some cases DNA fragments, by active sorting mechanisms, which can be uptaken by distant cells and transduce a message. EV is a generic term for vesicles secreted through different mechanisms: exosomes, microvesicles and apoptotic bodies. Exosomes arise from the fusion of an endosomal compartment, the multi-vesicular body (MVB), with the plasma membrane, while microvesicles and apoptotic bodies are secreted through shedding of the plasma membrane (for a review on exosome secretion mechanisms see ref.9). Because the biogenesis and sorting of the cargo involve different molecular machineries, exosomes and microvesicles have different contents and diameters (50–100 nm for exosomes; 10 to 1000 nm for microvesicles). The absence of specific markers and consensual purification methods account for the controversy on the nature of extracellular vesicles. Importantly,
recent data have demonstrated the existence of content heterogeneity within exosomes, suggesting the existence of multiple subpopulations of exosomes.\textsuperscript{10-13} Identification of new markers defining specific subtypes of EVs is therefore needed to understand their respective functions.\textsuperscript{12}

EVs play a pivotal role in cancer. Initially, EVs secreted by tumor cells were shown to induce an anti-tumoral immune response through the activation of dendritic cells and T lymphocytes.\textsuperscript{14,15} These studies initiated a new therapeutic approach using EVs as anti-tumoral vaccines (for review, see ref. \textsuperscript{16}). However, since this initial discovery, many studies have established that tumor EVs can promote tumor growth through different pro-tumoral activities. The current model suggests that tumor EVs, including exosomes, promote tumor growth and metastasis by modulating most of the hallmarks of cancer such as tumor cell proliferation, stromal cell differentiation, angiogenesis, the immune response and the properties of the surrounding extracellular matrix.\textsuperscript{17}

Tumor EVs are now being treated as potent promoters of metastasis and are used as promising targets in cancer diagnosis.\textsuperscript{18-20} Over the past couple of years significant efforts have been made to assess the functions of tumor EVs \textit{in vivo} and expand insights obtained from important yet limited \textit{in vitro} experimental studies. These new findings lead to a giant leap toward the understanding of the roles of tumor EVs in tumor progression, in particular in the evolution to metastasis.

Here, focusing on EVs secreted by tumor cells, we will first briefly describe their main functions identified through \textit{in vitro} experiments, by providing a few examples selected from the large number of publications in the field. We will then describe how the function of tumor EVs has been investigated \textit{in vivo} and how new molecular players contained in tumor EVs are emerging. Finally, we will describe recent imaging strategies, which now allow to follow EVs \textit{in vivo} and open new avenues to tackle open questions regarding their function in cancer progression.

**Tumor EVs \textit{in vitro}: Transferring malicious information to tumor, stroma, and beyond**

\textit{In vitro} experiments with EVs purified from tumor cells allowed the identification of several properties of tumor EVs, which, for some of them, have been confirmed \textit{in vivo} (see next section). In a seminal paper, Skog and colleagues demonstrated that human glioblastoma EVs contain functional pro-tumoral mRNA, miRNA and proteins, which can be endocytosed by endothelial cells and can promote angiogenesis \textit{in vitro}.\textsuperscript{21} They elegantly showed that recipient cells could efficiently translate EV-derived mRNA, providing a valid proof for their ability to deliver active information within the tumor microenvironment. The mechanisms by which cargoes are selectively enriched in EVs have started to be unraveled. Interestingly, sorting of miRNAs relies on the oncogene KRAS which is mutated in many human cancers.\textsuperscript{22,23} Besides, a recent study showed that cancer EVs are enriched in specific miRNA compared with non-cancer EVs. EVs carry cell-independent miRNA editing abilities, which could allow EVs to efficiently modify the phenotype of target cells by rapidly silencing selected miRNA.\textsuperscript{24} Importantly, further insights show that in a population of EVs the average number of copy of a given miRNA per EV is lower than one.\textsuperscript{25} Given the reported heterogeneity of EVs in terms of protein cargoes, this suggests that only a subpopulation of EVs is enriched in specific miRNA molecules and carry the functional miRNA message. Furthermore, while the transfer of miRNA activity from macrophages EVs to endothelial cells has been nicely demonstrated \textit{in vitro}, it was also shown to have a modest impact on target gene repression.\textsuperscript{26} Future methods allowing to track EVs-loaded miRNAs and assess their activity in the target cell will undoubtedly help solving the complex and heterogeneous nature of EVs.

In culture, tumor EVs can be ingested by tumor cells and confer them more robust tumoral traits. For instance, tumor cells can be transformed through the exchange of EVs containing the truncated and oncogenic form of the epidermal growth factor receptor (EGFR\textsuperscript{vIII}) in the case of glioma cells,\textsuperscript{27} or the oncoprotein KRAS in the case of colon cancer cells.\textsuperscript{28,29} Similarly, transfer of EVs between tumor cells can stimulate their proliferation and migration, as it has been shown for prostate cancer cells.\textsuperscript{30} Tumor EVs have also been shown to promote drug resistance \textit{in vitro}. Breast cancer cells exchange EVs mediating chemoresistance,\textsuperscript{31} and tumor cells can directly expel drugs via EVs.\textsuperscript{32-34}

\textit{In vitro} experiments suggest that tumor EVs can also be delivered to multiple types of stromal cells and thus modify their phenotypes. One of the most documented examples of this exchange is the effect of tumor EVs on endothelial cells. Tumor-derived EVs of different origins\textsuperscript{31,35-38} have the capacity to induce proliferation and tubulogenesis of endothelial cells \textit{in vitro} and could therefore promote angiogenesis during tumorigenesis. Interestingly, tumor EVs were also shown to destabilize the endothelial barrier and affect its permeability \textit{in vitro}.\textsuperscript{38-40} Besides, tumor EVs can be internalized by other cell types and induce their differentiation into potent and well-known pro-tumorigenic actors such as CAFs (Cancer-associated fibroblasts),\textsuperscript{41,42} myofibroblasts\textsuperscript{43-46} and macrophages.\textsuperscript{47} In addition, tumor EVs can also promote
immunosuppression in different ways. For instance, EVs purified from carcinoma or melanoma cell lines or purified from blood serum of patients with ovarian or colorectal cancer can induce the death of T-lymphocytes in vitro. Alternatively, EVs originating from different tumor cells can activate in vitro a population of cells, the myeloid-derived suppressor cells, known to suppress T-cell activation. Therefore, tumor EVs appear as potent immunomodulators presumably shaping tumor escape from immunosurveillance (for a review, see ref. 56).

In addition tumor EVs may also have an impact on the extracellular matrix (ECM) by either carrying and depositing ECM or by delivering ECM degrading enzymes such as MMPs. In a systematic study of the interaction between ECM and tumor EVs, EVs were shown to be enriched in several proteases, including active MMP2 and MMP9 that, efficiently degraded different molecules of ECM. Furthermore, the MT1-MMP metalloprotease is present in EVs secreted by different tumor cells and can efficiently degrade collagen or laminin in vitro. Recently, tumor EVs carrying MT1-MMP were shown to function in a coordinate fashion with invadosomes to promote matrix degradation and cellular invasion. Finally, EVs are capable of carrying important ECM molecules and deposit them in close vicinity to focal adhesions and thus inducing directional cell migration. In addition, fibronectin present at the surface of EVs mediates their interaction with heparin sulfate present on receiving cells, triggering the cell response.

Overall, these data demonstrate that tumor EVs have a functional impact in vitro and suggest that they could promote tumorigenesis by acting at different levels in vivo. Although these findings rely on the use of relatively high concentrations of EVs whose physiologic relevance has been questioned, they are now being supported by several evidences in vivo. Animal studies complement in vitro assays by allowing to follow EVs in their complex natural environment and, permit to assess their range of action, behavior in body fluids and their response to diverse challenges including blockade by epithelial/endothelial barriers, trapping by ECM and depletion by clearance mechanisms.

**Pro-tumoral role of tumor EVs in vivo**

Studies describing the function of tumor exosomes in vivo fall into 2 categories. In a first category of experiments, genetically engineered tumor cells allowing tuning of EV secretion levels are injected into mice and their capacity to form metastasis is assessed (Fig. 1A). Using

![Figure 1](image-url)

**Figure 1.** Functional analysis of tumor EVs. (A) The impact of tumor-secreted EVs on tumor growth and metastasis relies on the amounts of EVs and their loading in specific RNA or protein cargoes. Here, expression levels of genes involved in EV biogenesis or encoding EV cargoes are tuned (si/shRNA or overexpression) in tumor cells, which are grafted into mice. Subsequent tumor growth (T) and metastasis (M) formation is tracked over time. (B) Alternatively, isolated EV (where expression of cargoes has also been tuned) are used for priming a metastatic niche. For this, repeated intravenous injection of EVs purified from cultured tumor cells are performed, favoring the formation of a pre-metastatic niche. Metastasis onset and growth is then assessed upon subsequent intravenous injection of tumor cells.
this approach, it was demonstrated that depletion of Rab27A in mammary carcinoma or melanoma cells decreases the levels of secreted EVs in vitro and hampers in vivo tumor growth and metastasis.\textsuperscript{45,64,65} Exosomes isolated from advanced metastatic melanoma were shown to carry high concentrations of TYRP2, VLA4, HSP70, MET and Rab27a proteins and could instigate BDMCs (Bone marrow-derived cells) to secrete increased levels of MET thereby favoring the establishment of a pro-metastatic niche.\textsuperscript{64} In mammary carcinomas, secreted EVs, together with soluble proteins, repress the tumor-prone and systemic accumulation of neutrophils.\textsuperscript{65} These studies allowed the identification of Rab27A as a key driver of EV biogenesis and tumorigenesis. Surprisingly however, although overexpressing Rab27A expectedly leads to increased secretion of exosomal proteins in a lung cancer cell line, in vivo immunization of mice with such EVs leads to reduced tumorigenesis by eliciting an antitumoral immune surveillance.\textsuperscript{66} Whether overexpressing Rab27a also increases the amount of secreted EVs is not clear and needs to be determined. The role of Rab27b, originally involved in EV expressed in EV from Hela cells,\textsuperscript{67} is more complex. Whereas its depletion does not affect EV secretion in 2 mammary tumor cell lines,\textsuperscript{65} Rab27b overexpression increases the secretion of HSP90, a known marker of EVs, and stimulates breast tumor growth, invasion and metastasis.\textsuperscript{68}

Two other RAB GTPases RAB22A and RAB3D, known to regulate EV formation, have been similarly used to determine the role of EVs in tumor progression.\textsuperscript{69,70} Depletion of RAB22A in MDA-MB231 breast cancer cells decreases their capacity to secrete EVs under hypoxia in vitro and impairs their ability to form lung metastasis in mice.\textsuperscript{69} RAB3D overexpression increases EVs secretion in breast cancer cells in vitro, and in parallel promotes breast cancer cell invasion and lung metastasis in mice, while its downregulation has the opposite effect.\textsuperscript{70} Similarly, breast cancer cells overexpressing the neutral Sphingomyelinase nSMase2 secrete more EVs in vitro and develop more lung metastasis through an increase in angiogenesis once injected in mice, while cells depleted for nSMase2 have an opposite phenotype.\textsuperscript{71,72} Together, these studies generally correlate the levels of secreted EVs by tumor cells and the capacity of these cells to form tumors and metastasis in mice. However, one important drawback of this approach is that all the genes known to affect EV secretion so far also have other cellular functions, which can contribute to cancer progression independently of EV secretion.

In the second category of studies, EVs are purified from cultured tumor cells and subsequently injected into animals (before or together with tumor cells), before assessment of tumor growth and metastasis (Fig. 1B). For instance, repeated intraperitoneal injection of exosomes purified from mammary tumor cells before subcutaneous injection of tumor cells in the mammary gland, enhances tumor growth, through inhibition of immune NK cell growth and activity.\textsuperscript{73} Similarly, EVs purified from rat pancreatic adenocarcinoma or from human renal carcinoma cells promoted lung metastasis of tumor cells in rats\textsuperscript{74} or mice.\textsuperscript{36} Also, orthotopic co-injection of human mammary tumor cells with exosomes from breast cancer patients, enhanced tumor growth in nude mice which was not seen with EVs from healthy individuals.\textsuperscript{24} Very recently, melanoma EVs repeatedly injected in the circulation were shown to ‘activate’ alveolar epithelial cells, potentially through non-coding small nuclear RNAs, thereby favoring the neutrophil-dependent education of PMN.\textsuperscript{75} Over the past couple of years, this strategy has notably been used to identify a new role for tumor EVs in preparing a pre-metastatic niche at distance from the primary tumor.\textsuperscript{36} Using different models, the group of David Lyden has shown that injection of tumor exosomes in the mouse circulation modifies the microenvironment of specific organs, notably in the absence of tumor cells.\textsuperscript{64,76,77} In a seminal study by Peinado and colleagues, lethally irradiated mice were transplanted with bone marrow from GFP-expressing mice that were previously treated with exosomes purified from melanoma cells.\textsuperscript{64} Upon bone marrow reconstitution, growth, angiogenesis and metastatic potency of orthotopic tumors were enhanced in mice whose bone marrow had been ‘educated’ by tumor EVs (versus synthetic vesicles or PBS).\textsuperscript{64} This groundbreaking discovery was later confirmed using pancreatic cancer EVs, whose injection in mice leads to a stroma-dependent fibrotic niche, to the benefit of colonizing tumor cells.\textsuperscript{76} Here, exosomes from pancreatic tumor cells were shown to be loaded with MIF (Migration Inhibitory Factor) that drives a pro-fibrotic secretion of cytokines, such as TGF-ß, by targeted Kupffer cells leading to a pro-metastatic deposition of Fibronectin by local Hepatic Stellate cells.\textsuperscript{76} Similarly, injection of cancer EVs can modify the phenotype of fibroblasts in the lungs, or affect Kupffer cells in the liver, depending on the cellular origin of the EVs and their integrin repertoire at their surface.\textsuperscript{77} Altogether, these experiments point to a model where tumor cells secrete EVs, which can be ingested by stromal cells either locally or at distance. This will induce local alterations in the tumor microenvironment as well as generate a metastatic niche at distant sites. More specifically, tumor EVs can modulate the immune response, modify the ECM or alter the phenotype of stromal cells. Although very informative, these studies would strongly benefit from in vivo tracking of EVs, using reliable
markers and state-of-the-art intravitral imaging, with a resolution adapted to EVs (see last section). Nevertheless, these studies paved the ground for the identification of important EV cargoes in cancer progression.

**Function of individual EV cargoes in tumor progression**

Over the past couple of years, several tumor EV cargoes, miRNAs and proteins, have emerged as essential modulators of the function of tumor EVs. Two factors present in tumor EVs, the protein Sem3A and the miRNA miR105, were shown to promote endothelial permeabilization, thereby favor tumor metastasis.\(^{38,40}\) The transmembrane protein Semaphorin3A, when present at the surface of glioblastoma stem cell EVs, promotes vascular permeability in the murine brain by destabilizing VE-cadherin.\(^{40}\) The microRNA miR105, which is only expressed by metastatic breast cancer cells, localizes in EVs and induces a destabilization of the endothelium through a disruption of tight junctions via ZO1 downregulation, thus promoting metastasis in mice.\(^{38}\)

In addition to miR105, several miRNAs present in tumor EVs are now known to functionally contribute to tumor progression *in vivo* through distinct processes. EV transfer of miR122 to stromal cells reprograms glucose metabolism in PMN in mice, thereby promoting metastasis,\(^{78}\) while miR155 delivered by the tumor EVs promotes drug resistance in a neuroblastoma model.\(^{79}\) Meanwhile, two other microRNAs, miR192 and miR29a/c, were shown to have an opposite effect and decrease tumor progression when present in tumor EVs.\(^{80,81}\) miR192, whose expression is lower in highly metastatic human adenocarcinoma cells, inhibits angiogenesis *in vitro* when overexpressed in EVs. Repeated injection of EVs containing miR192 in mice represses osseous colonization.\(^{80}\) miR29a/c, which can target VEGFR, can be transferred to endothelial cells via EVs *in vitro*, and subsequently decreases angiogenesis. Treatment of mice with intravenous injections of miR29a/c EVs inhibits tumor growth and angiogenesis *in vivo*\(^{81}\) which may suggest therapeutic opportunities.

In addition, several transmembrane proteins, present at the surface of tumor EVs, contribute to their function and to their distribution in the organism. The tetraspans Tspan8 and CD151 promote PMN formation in a rat model of pancreatic adenocarcinoma and promote metastases in lungs and bone marrow, most likely through extracellular matrix remodeling.\(^{82}\) Interestingly, EVs deprived of Tspan8 have a reduced capacity to cross the blood-brain barrier *in vivo*. Finally, integrins, which are transmembrane proteins better known for mediating important cell-ECM and cell-cell contacts, are present on the surface of EVs and were shown to dictate the organotropism of tumor EVs and subsequent metastasis in mice.\(^{77}\) Indeed, while tumor EVs derived from pancreatic or breast cancer cells, carrying integrins α6β4 and α6β1 specifically target lungs, EVs with the αvβ5 integrin specifically target the liver.

Successful identification of important cargoes often relies on the ability to track labeled EVs in the animal, which is a challenging task due to their small size, absence of bonafide markers and the lack of adapted imaging technologies. Therefore, recent development in EV tagging, tracking and imaging technologies is largely advancing the field of tumor EVs biology.

**Following tumor EVs *in vivo***

Following EVs in living multi-cellular organisms is challenging, due to the small size of these vesicles and to their rapid dispersion in body fluids. Different strategies have been developed to label EVs, allowing to follow them in the animal at different scales (from whole body to cellular level) and with different specificities (global labeling of EVs or specific targeting of subpopulations) (Fig. 2, Table 1). Two approaches have been used to track, at the animal scale, large amounts of EVs injected at once in the circulation of mice: bioluminescence and radiolabeling. In the first case, the luciferase is directed to EVs, either randomly\(^{83}\) or through a fusion with a transmembrane peptide\(^{84,85}\) while in the second case, radiolabeling is achieved after EV purification, either in subpopulations of EVs expressing a specific fusion protein\(^{86}\) or in the global population of EVs through the introduction of radiolabeled probes.\(^{87}\) These approaches allow the non-invasive tracking of EVs at the animal scale and identification of the tissues that received most EVs, although they lack further resolution.

Alternatively, many studies have used fluorescent lipophilic dyes. In particular long chain carbocyanines such as PKH, Dil or DiR, which insert into the EVs lipid bilayer, have been used to label EVs post-purification (examples: refs. 77, 85, 88, 89). However, their labeling specificity and reliability over time has been questioned\(^{84,86}\) More specific labeling can be obtained through the expression of fluorescent proteins fused to proteins or peptides present within the EVs (such as CD63, the C1C2 domains of lactadherin or a palmitoylation signal) in the secreting cells.\(^{90,92}\) However, this approach restricts the labeling to a subpopulation of EVs, may create artifacts due to overexpression of the marker. In addition, the signal intensity of this fluorescent fusion protein depends on the expression levels of the reporter protein.\(^{84,86}\) Further studies will be needed.
for designing methods and live-compatible dyes capable to reliably and stably label distinct EV populations.

Finally, methods have been developed to follow the fate of mRNA cargoes transported by EVs. These include luciferase mRNA, which will be translated by the receiving cell and therefore label it in an enzymatic assay \(^90\) or Cre mRNA,\(^83,93,94\) whose translation can be observed in the receiving cells using lox-reporter transgenic mice or cells. This latter approach turned out to be a very potent tool to identify the tumor EV receiving cells \(^93,94\).

All these different approaches provided a first insight into the behavior of tumor EVs in vivo. Interestingly, although EVs seem to be extremely stable in body fluids ex-vivo,\(^84,95\) they are cleared from the circulation in only a few minutes following their intravenous injection in mice.\(^85,86,96\) The biodistribution of EVs in organs varies between studies, as they have been reported to mainly reach the spleen, liver, lungs, bladder or kidneys. In addition, EVs injected intradermally in the mouse tail rapidly reach the lymphatic vessels and accumulate in the lymph nodes where they are stably retained for a couple of days, in addition to other organs (kidney, liver and thymus).\(^97\)

These differences could be due to the distinct cellular origins of the EVs, the different labeling methods or the different modes or doses of administration.\(^84,98,99\) Similar discrepancies have been reported when testing the tumor-targeting potential of tumor EVs injected in animals already hosting a mammary tumor. In particular, it has been reported that these EVs are massively incorporated in the tumor,\(^84,89\) suggesting a strong therapeutic potential,\(^89\) while another study showed only minimal accumulation in tumors.\(^87\)

Although these studies have brought important insights in the biology of EVs, they rely on the bulk injection of tumor EVs in the circulation, which might not necessarily represent what happens upon natural progressive release of EVs by a tumor.

The identity of the cells incorporating tumor EVs is a central question. Several studies have demonstrated the existence of a cell specific uptake in vitro (for instance\(^100,101\)). Several in vivo studies demonstrate that EVs of different origins are mainly incorporated by specialized phagocytic cells, such as dendritic cells or macrophages in mice.\(^77,94,96,97,102-104\) This is the case for instance of pancreatic cancer EVs injected in the circulation, which are uptaken by Kupffer cells in the liver.\(^76\)

Strikingly, tumor EVs are much more stable in the circulation and in several organs, once macrophages have been depleted in mice, demonstrating that macrophages...
might be the main cell type absorbing tumor EVs upon bulk injection. It will be important to confirm whether these cells are also targeted by EVs released in physiologic levels. This however requires first the establishment of stable and genetically-encoded EV markers in relevant in vivo models. Nevertheless, other cell types have been shown to incorporate tumor EVs in vivo, including endothelial cells, alveolar epithelial cells, fibroblasts, neurons and microglia and tumor cells themselves. The molecular mechanisms underlying potential tumor EV organotropism and cell-type specific uptake, start to be unraveled where integrins seem to play an important role. The fate of uptaken EVs (degradation vs signaling) and the functional consequences of this uptake on the receiving cell phenotype and on cancer progression deserve particular attention.

The secretion of EVs by tumor cells and their transfer to stromal cells has been witnessed in vivo in recent groundbreaking studies using intravital imaging, opening the door to the dynamic analysis of such transfer. Using time-lapse multiphoton imaging, the group of X. Breakfield has been able to follow tumor EVs labeled with a palmitoylation signal fused to GFP (Palm-GFP) that had been secreted either by glioblastoma (using a cranial window) or by thymoma cells (using a dorsal skinfold window) in living mice. In both cases, tumor EVs were mostly stationary in the center of the tumor and rather mobile on its edges, where interactions and exchange with stromal cells could be visualized. Meanwhile, Zomer et al. used an elegant genetic approach to follow the fate of tumor EVs containing Cre mRNA in the living mouse. This demonstrated the transfer of mRNA contained in EVs secreted by carcinoma and melanoma cells to tumoral cells (both local and systemic exchanges) and non-tumoral cells (immune and non-immune) as well as the transfer of migratory and metastatic properties. It is important to note that these studies have essentially described rather large EVs in vivo. Whether this is due to the labeling methods used, or to the limits of optical resolution, is not clear.

The recent studies described above demonstrate that combining different labeling and imaging strategies to follow EVs naturally released by a primary tumor in a living animal provides unique insights into their behavior and function in vivo. It could for instance allow to follow the natural dispersion of tumor EVs from a primary tumor, which might lead to different observations than upon bulk injection of EVs in the circulation. This has been recently illustrated by Pucci et al. who combined multiple strategies of EV labeling and imaging to study tumor EVs released by melanoma in mice. They showed that most EVs naturally released by tumor cells end up in the lymph nodes where their uptake by macrophages inhibits tumor growth, by preventing them to interact with B-cells. Using similar combination of strategies will certainly allow to better describe the fate and function of tumor EVs in a near future.

Another exciting field of investigation that could be approached using pioneer imaging technologies concerns the cellular mechanisms of secretion, uptake and sorting of tumor EVs in vivo. Determining the fate of tumor EVs in receiving cells, in particular identifying in which compartment they are sorted, will be instrumental in understanding how their message is delivered. Besides, intriguing mechanisms have recently been described in vitro, but their existence in vivo is so far unknown. It has for instance been shown that exosome secretion is coupled to the formation of an actin rich protrusion involved in tumor cell invasion, the invadopodia. Moreover, EVs were shown to be taken up by filopodia from the receiving cell, shuttled in endosomes where they scan the endoplasmic reticulum before being directed to lysosomes. The precise characterization of this mechanism relied on the use of the exosomal tetraspanin CD63 fused to GFP to monitor its behavior by time-lapse confocal microscopy and to the APEX2 tag allowing to precisely locate it by electron microscopy. Ideally, the study of EVs at the cellular to subcellular scale would require intravital Correlated Light and Electron Microscopy (CLEM). This challenging approach, which allows to follow an event at the fluorescent scale in the living animal and retrieve it at electron microscopy scale, could permit the precise characterization of tumor EV secretion uptake and sorting. There is no doubt that applying this methodology to relevant animal models mimicking the metastasis cascade will unravel the exact subcellular mechanisms underlying the delivery of exosome-carried messages.

**Outstanding open questions**

In a near future, the imaging strategies described in the paragraph before will certainly help to address general open questions, such as how EVs cope with the extracellular matrix, how they cross the endothelial barrier or how they behave in fluids such as the blood and lymphatic circulation. These issues need to be resolved to fully understand the relevance of tumor EVs in cancer progression. They could benefit from alternative model organisms, which allow nice intravital imaging and constitute emerging cancer models in vivo, such as Drosophila (where EVs function has been described in a developmental context). In addition, a recent study has used the zebrafish embryo model for testing their potential in drug delivery. Although preliminary, these studies suggest that the zebrafish embryo could be a very useful model for studying the biology of exosomes in the near future.

In addition, several important questions regarding tumor EVs remain to be addressed in vivo. While in vitro studies
Table 1. Summary of the different approaches used to study tumor EVs in vivo in mice. Representative references are given as examples.

| Approach | Assay type | Details | Advantages | Disadvantages | Refs |
|----------|------------|---------|------------|---------------|------|
| Formation of tumors secreting different levels of EVs | Functional | Modulation of genes regulating EVs secretion (e.g.: RAB27a, RAB22A, nSMase2, RAB3D...) | “Endogenous” release of EVs (by grafted tumors) & Tumor growth and metastasis formation in their relevant microenvironment | Potential side-effects: target genes can have EV-independent functions impacting on tumor growth and metastasis | 45, 64, 65, 69, 70, 72, 104 |
| Tumor secreting EVs with different contents | Functional | Modulation of an EV protein or miRNA (e.g.: Sema3A, Integrin, miR105, miR122...) | “Endogenous” release of EVs (by grafted tumors) & Relevant tumor microenvironment | Blind approach: visualization of secreted EVs is impossible | 38, 40, 77, 78, 79, 80, 82 |
| Tumor secreting fluorescent EVs | Descriptive | Genetically-encoded proteins or peptides secreted into EVs | “Endogenous” release of EVs (by grafted tumors) & Relevant tumor microenvironment & Allows intravital imaging (dynamic) & Traduction of mRNA encapsulated in EVs can be followed | Fluorescent EVs only represent a selected population of EVs and depends on the marker used | 38, 40, 77, 78, 79, 80, 82, 106 |
| Tumor secreting EVs containing Cre mRNA | Descriptive | Tumor EVs contain Cre mRNA | “Endogenous” release of EVs (by grafted tumors) & Relevant tumor microenvironment & Allows intravital imaging (dynamic) & Visualization of secreted EVs is impossible, except for translated mRNAs | Blind approach: visualization of secreted EVs is impossible | 83, 93, 94, 104 |
| Injection of tumor EVs | Injection of EVs together or before injection of tumor cells | Functional | Modulation of the cellular origin of the EVs, or modulation of an EV protein or miRNA | Effects observed only depend on the injected material & EV content can be manipulated | Bulk injection of EVs might not reflect normal secretion features (mode of entry into the circulation, levels, timing) | 24, 36, 64, 73, 74, 75, 76, 77, 80, 81 |
| Bio-luminescent labelling of EVs | Descriptive | Organism scale & Longitudinal analysis & Global labelling: all EVs are labelled | Lack of resolution | 83, 84, 85 |
| Fluorescent lipid Dyes | Descriptive | | | Specificity of labelling | 76, 77, 85, 87, 88, 89 |
show that more aggressive cancer lines secrete more EVs (e.g.,\textsuperscript{64}), it is currently not clear whether tumor cells continuously secrete similar levels and populations of EVs, or whether they vary following tumor progression. Similarly, tumors have been shown to be composed of heterogeneous populations of cells, but how this is linked to different amounts of EVs and EVs with different content is unknown \textit{in vivo}, although there are some hints from \textit{in vitro} studies.\textsuperscript{117} Furthermore, the heterogeneity of EVs, which has been demonstrated \textit{in vitro}, still awaits validation \textit{in vivo} and the functional significance of this diversity remains to be addressed. A recent study used intravital lung imaging in mice to show that melanoma cells arriving in the lung capillaries release large cytoplasmic blebs (5 \(\mu\)m average diameter), which are uptaken by myeloid cells and promote the formation of a successful metastasis.\textsuperscript{118} This study reveals that exchange of cellular material cellular can occur via diverse mechanisms, going beyond small extracellular vesicles, and thereby control the efficient formation of metastatic niches \textit{in vivo}.

Importantly, stromal cells also secrete EVs which contribute to tumor progression, as it has been shown for EVs secreted by stromal cells,\textsuperscript{119,120} and astrocytes.\textsuperscript{121} Therefore, EVs mediate the communication between tumor and stromal cells in both directions and it might be interesting to determine how one influences the other, through positive or negative feedback loops.

Finally, future studies should aim at identifying cargoes whose function could be exploited for the design of effective therapeutic approaches. Understanding the involvement of individual EVs cargoes \textit{in vivo} could benefit from a recently developed tool allowing a temporal control of cargo loading in EVs through optogenetics which functions \textit{in vivo}.\textsuperscript{122} There is no doubt that the fascinating and fast evolving field of extracellular vesicles has been booming over the past years. Nevertheless, this field requires the design of studies dedicated to refine the role of EVs in cancer progression and to validate their potential targeting in anti-tumor therapies. Those aspects would therefore strongly benefit from establishing relevant \textit{in vivo} models, experimental strategies, adapted imaging approaches and associated quantification of EV levels and delivery according to tumor stage and progression.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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