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## Spitting out the demons: extracellular vesicles in glioblastoma

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### Abstract

Discovered decades ago, extracellular vesicles (EVs) emerge as dedicated organelles, able to deliver protected, specific cellular cues throughout the organism. While virtually every cell can release EVs, cancer cells co-opted this feature and efficiently unleashed them both in the tumor microenvironment and towards healthy tissues. This might contribute to tumor aggressiveness and spreading. Cancer-derived EVs that contain DNA, mRNA, miRNA, and packed and transmembrane proteins can operate locally or at distance. This review will focus on the high-grade brain tumor (i.e. glioblastoma)-derived EVs, discussing recent reports on i) their phenotype and content, ii) their putative functions, and iii) their clinical potential for improving diagnosis and therapeutics.

**Keywords:** glioma, exosomes, angiogenesis, permeability, brain tumors, signaling, tumor microenvironment

## **Introduction**

Although rare, glioblastoma (GBM) represents the most malignant and deadly brain tumor in adults, with a poor prognosis despite intensive researches and clinical efforts. <sup>1</sup> GBM exhibit high proliferation rate, invasion, angiogenesis and necrosis because of the diffuse and aggressive nature of the tumor cells. Relapse is almost inevitable and fatal within a short window of 7-10 months, while the average survival does not exceed 18 months following diagnosis. Current standard treatments are essentially palliative, typically involving surgery followed by radiotherapy and DNA-alkylating chemotherapeutic agents to eliminate the remaining cells. <sup>1</sup> Meanwhile, a subpopulation of cells with tumor-initiating properties bears resistance to conventional therapies, and as such has been implicated in tumor recurrence. <sup>2</sup>

Numerous recent studies unveil that tumor cells can efficiently release selected intracellular content in the milieu, embedded and protected in lipid layer-based structures, named extracellular vesicles (EVs). Such tumor-derived EVs may contribute to intercellular communication, tumor progression and resistance mechanisms in the context of GBM. <sup>3</sup>

## **Extracellular vesicles in glioblastoma**

### **General definition of the tumor-derived EVs**

Extracellular vesicles (EVs) were discovered over 30 years ago and defined as extracellular lipid bilayer spherical structures from 30 to 1000 nm that are secreted in the cellular environment. <sup>4,5</sup> An international consortium adopted the generic term of extracellular vesicles (EVs) to clarify between the different names used in the literature. <sup>6</sup> The main subcategories of EVs are classified according to their size and their subcellular origin, while their content appears rather versatile. Classically, exosomes exhibit diameters of 30-100 nm and mostly originate from intra-cytoplasmic vesicular bodies; microvesicles (or ectosomes) range between 100 nm to 1  $\mu$ m diameter and are derived from the plasma membrane; apoptotic blebs are the largest population of EVs, with a diameter of approximately 1-2  $\mu$ m and emanating from dying cells. <sup>7,8</sup> Recently, the term of oncosomes was coined to define large EVs (1 to 2  $\mu$ m) arising from the budding of the plasma membrane in cancer cells. <sup>7,8</sup>

Studies in the 1970's reported cell membrane undulations and peripheral vacuoles bordering the extracellular space of GBM giant-cells,<sup>9</sup> suggesting that tumor cells could be a non-physiological source of extracellular lipid layer-based structures. More recently, abundant EVs were observed on the surface of primary human GBM cells in culture.<sup>10</sup> Interestingly, it has been estimated that one primary GBM cell releases about 10,000 EVs *in vitro* over a period of 48 hours.<sup>10,11</sup> Molecularly, the fact that tumor cells exhibit enhanced EVs release as opposed to normal cells could be intrinsically caused by oncogenic mutations. For instance, the EGF receptor variant III (EGFRvIII), a feature of nearly 50% of adult GBM was shown to augment GBM cell-derived vesiculation.<sup>12</sup>

### **The molecular composition of EVs in glioblastoma**

Tumor-based EVs can carry a wide range of molecules from nucleic acids (including genomic and mitochondrial DNA, mRNA, miRNA) to proteins and lipids that mirror tumor cells and state.<sup>13</sup>

In 2008, Skog et al were among the first to report that GBM-derived EVs contain mRNA and miRNA.<sup>10</sup> They showed that the EV content is selectively enriched, as compared to the parental cells. Likewise, miRNA can be conveyed within GBM-released EVs.<sup>15,16</sup> In that view, miR-21, known for its protective action on GBM cells<sup>17</sup> is enriched in GBM-shed EVs.<sup>10</sup> Conversely, genomic or mitochondrial DNA were rarely reported in GBM-derived EVs.<sup>11,18</sup> Proteins are also specifically sorted and embedded in EVs, although the packaging mechanism is not yet elucidated (for a review, please see ref. 8 and 19). Mass spectrometry analysis of GBM-derived EVs identified more than 100 proteins that are also usually found in exosomes, based on the ExoCarta database.<sup>15,20</sup> Interestingly, cytokines are enriched in GBM-produced EVs, such as pro-angiogenic agents, among which VEGF-A,<sup>10</sup> the pro-permeability guidance molecule Semaphorin 3A,<sup>21</sup> and the immunosuppressive cytokine TGF- $\beta$ .<sup>22</sup> Additionally, one of the best-characterized GBM-based EV-harbored proteins is the tumor-specific EGF mutated receptor EGFRvIII.<sup>10,12</sup> Finally, whereas lipids are structural components of EVs, there is a lack of data on lipid composition<sup>23</sup> and rare information as for GBM.

An important layer of complexity arose from the heterogeneity of EVs content produced by GBM tumors. Briefly, four different GBM subtypes, namely proneural, neural, classical and mesenchymal, have been described according to their molecular signatures.<sup>24</sup> Importantly, GBM-derived EV protein content remain clustered within

the same subtype of their origin (i.e. with proneural or mesenchymal signatures),<sup>25</sup> suggesting that hallmarks of each subtype is conserved and transferred throughout GBM-derived EVs even if the exact cargoes remain unknown. As a consequence, GBM-produced EVs may exhibit the ability to disseminate oncogenic materials.

### **EV uptake and diffusion**

Among the communication network that operates between cancer cells and their environs (i.e secretion, cell-cell junction, mechanical forces, tunneling nanotubes, *etc*), EVs represent a powerful tool. EVs are released locally into the extracellular tumor microenvironment and are able to spread throughout the organism, as GBM-derived EVs can be found circulating into the bloodstream, urine and cerebrospinal fluid.<sup>26,27</sup> The molecular and cellular mechanisms of uptake, including endocytic mechanisms, ligand/receptor interaction and fusion with plasma membranes will not be discussed here (for a review, please see ref. 7).

In GBM, neighboring cells such as healthy neural cells, endothelial cells, microglia, monocytes/macrophages and tumor cells have been documented to uptake tumor-derived EVs (*Table 1* and please see our next section).<sup>10,21</sup> Indeed, mRNA materials identified in GBM-derived EVs could be transferred to surrounding recipient cells.<sup>10,14,15</sup> Moreover, specific mRNA mutations (i.e. IDH1) were detected in EVs isolated from glioma patient sera and cerebrospinal fluid.<sup>29</sup> As *IDH1* mutations are well described in a subset of GBM patients,<sup>30,31</sup> we can hypothesize that these circulating EVs, which contain specific mutated mRNA, may arise directly from GBM cells. Recently, intravital imaging unveiled that GBM-derived EVs are ingested by the surrounding microglia and monocyte/macrophages *in vivo*.<sup>32</sup>

Interestingly, electronic microscopy analysis unveiled the presence of nanofilaments on exosomes derived from U87 and U251 GBM cell lines, but not on normal astrocytes.<sup>28</sup> As such, it is tempting to speculate that this phenotypic observation could have functional consequences. For instance, such nanofilaments may create a diffusive network around cancer cells and participate in the direct fixation of secreted exosomes to their cellular targets.<sup>28</sup>

Thus, GBM-released EVs might ultimately contribute to tumor progression and heterogeneity, since they retain their ability to be taken up by multiple cellular targets and to disseminate throughout the body.

## Functions in the brain tumor microenvironment and beyond

### EVs action within brain tumors

In the central nervous system, the main postulated physiological role for EVs corresponds to their ability to rapidly and robustly exchange signals between glia and neurons, thus promoting neuronal survival, microglia-mediated immune responses, and synapse assembly and plasticity.<sup>33</sup> This system is most likely corrupted in brain tumors to the tumor's own benefit. Indeed, EVs represent important means of communication within the tumor microenvironment and GBM-derived EVs have been suggested to promote cell proliferation, invasion, immunotolerance, angiogenesis and endothelium defects (*Figure 1*).

It has been shown, for example, that hypoxic conditions can increase the hypoxic-related mRNA and protein content of tumor-released EVs, which in turn impacts on tumor growth both *in vitro* and *in vivo*.<sup>14</sup> The analysis of these patient-derived exosome-like fractions revealed that the enriched mRNA and proteins were associated with poor prognosis,<sup>14</sup> suggesting that cancer cells can adapt to the hostile hypoxic microenvironment. Conversely, EVs also convey messages to hack their environment. MiR-1 overexpression in glioblastoma stem-like cells (GSC) was able to modify EVs protein cargoes and to ultimately reduce tumorigenicity, invasiveness and angiogenesis *in vivo* in xenografted mice.<sup>34</sup> From a molecular standpoint, miR-1 targets the mRNA of Annexin A2, one of the most abundant proteins in GBM-derived EVs, in addition to other pro-oncogenic signals.<sup>34</sup> In keeping with this idea, GBM-produced EVs were shown to operate directly on endothelial cells to promote tubulogenesis and permeability, two hallmarks of tumor angiogenesis.<sup>25,35</sup> Likewise, EVs collected from GBM cell lines can transfer RNA to normal brain endothelial cells.<sup>10</sup> Moreover, our team has demonstrated that GSC-liberated EVs convey the pro-permeability guidance molecule semaphorin 3A towards neighboring brain endothelial cells and ultimately contribute to enhance vascular permeability in orthotopic GBM xenograft models,<sup>21</sup> reinforcing the concept that tumor-derived EVs can pervert the vasculature. In line with the hypothesis that EVs play a role in the sabotage of their environment, GBM-produced EVs can be taken up by innate immune cells, microglia and monocytes/macrophages within the brain, and shift their cytokine expression profile and mRNA content towards a pro-tumoral phenotype (*Figure 1*).<sup>32</sup>

Although these recent compelling data highlight the likely involvement of GBM-derived EVs to tumor properties, we have to keep in mind that most of these observations occurred following administration of EVs collected after several steps of purification/concentration and/or from *in vitro* experiments. Interestingly, intravital imaging was recently deployed to directly image GBM-derived EVs transfer to microglia and monocytes/macrophages within the brain in living animals. As a consequence, recipient cells were denatured, as exemplified by changes in the levels of miR-21 and c-MYC RNA.<sup>32</sup>

### **Is there a role for circulating EVs?**

EVs emanating from the tumor mass have been detected outside the tumor microenvironment, in particular circulating freely in the plasma.<sup>21,26,27</sup> While this feature could be employed as a diagnostic tool (please see our next section), the functional and biological consequences remain unclear in GBM (*Figure 1*). In the context of melanoma, endogenous tumor-derived EVs were tracked *in vivo* by the means of multiple reporter mouse strains, and their further dissemination through lymph nodes was visualized and established to precede tumor cell detection.<sup>36</sup> This study established that circulating tumor-derived EVs could also reshape at distance systemic responses, and affect in turn tumor progression.

In GBM, two recent studies suggested that circulating EVs can modify the immune system and contribute to immunomodulation in systemic and in the tumors.<sup>37</sup> First, GBM-derived EVs impact on the monocytic lineage, which acquires a *de novo* tumor-supportive phenotype.<sup>37</sup> Additionally, EVs derived from serum-purified GBM patients were shown to promote the M2-like phenotype, with the serum cytokine profile typical of Th2 bias, suggesting an action of EVs throughout the organism.<sup>38</sup>

Paralleling the action on the immune system, GBM-derived EVs can affect the biology of the vasculature. For instance, thromboembolic diseases observed in GBM patients might rely on the pro-coagulant activity detected in circulating microparticles.<sup>39,40</sup> Circulating EVs were shown to transport pro-permeability and pro-angiogenic factors,<sup>10,21</sup> including GSC-derived semaphorin 3A that could reach the endothelium of virtually all healthy organs.<sup>21</sup> Indeed, GBM patient serum-purified EVs are loaded with semaphorin 3A, which further orchestrated the loss of barrier integrity.<sup>21</sup> One important challenge will be to track *in vivo* specifically GBM-derived EVs and

explore both their cargoes and their biological functions, in the course of tumor initiation, progression and relapse.

## **Translational outcomes and clinical potential**

### **Could EVs serve as prognosis/theranostic marker?**

Lack of therapeutically convenient monitoring tools remains a major cause of failure in the management of GBM patients. <sup>41</sup>For example, MRI only detects already established tumor of several hundreds or thousands of tumor cells. <sup>42</sup> According to the recent review by Westphal and Lamszus, <sup>43</sup> there are still only very few clinically relevant markers for GBM, among them MGMT methylation status, 1p/19q codeletion in oligodendroglial tumors, the EGFRvIII variant, and IDH1 and BRAF mutations. <sup>43</sup> Interestingly, analysis of EVs derived from biofluids emerged as a promising source of biomarkers because: i) they are easy to collect and implied non-invasive procedures as opposed to intracranial tissue biopsies; ii) their content mirrors the genetic and cellular status of mother tumor cells; <sup>13</sup> and iii) the half-life of labeled EVs seems quite short in the blood circulation, suggesting that they could be a suitable biomarker reflecting rapid changes in tumor cell state (*Figure 2*). <sup>44</sup> This raises the possibility that several biomarkers could be combined to recapitulate the tumor heterogeneity at a given time point and in the course of tumor progression, from multiple exploitable clinical samples (blood, urine, cerebrospinal fluid, saliva, lymph, sexual secretion *etc*).

The translational interest for GBM diagnosis was firstly highlighted with the pioneer work of Skog et al. <sup>10</sup> In this study, they demonstrated that EVs purified from patient sera, as opposed to healthy donors, are enriched in a specific subset of RNA (i.e. EGFRvIII and miR-21) that could be further used to monitor oncogenic mutations and gene expression. <sup>10</sup> In keeping with this idea, Akers et al. <sup>45</sup> characterized the presence of miR-21 in EVs prepared from cerebrospinal fluid of GBM patients. However, the in-depth determination of the relative abundance of miRNA in plasma- or cerebrospinal fluid-isolated EVs reveals the heterogeneity of such biomarkers. <sup>46</sup> Indeed, this study reported that the amount of detectable miRNA was rather low and heterogeneous in plasma exosomes or microvesicles, with an average of less than one miRNA molecule monitored per 200 to 85.000 EVs. In contrast, this average was higher in cerebrospinal fluid and increased to around one molecule of miRNA for 150--25.000

EVs. <sup>46</sup> This emphasizes that extraction protocols and detection sensitivity are critical steps for robust clinical applications. <sup>47,48</sup> Optimizing preservation of EVs content and improving quantification methods are currently under investigation to determine new informative markers. <sup>46,47,49</sup> Furthermore, a number of recent studies established that, in addition to mRNA and miRNA, proteins can also be examined from plasma-isolated EVs. <sup>21,50</sup>

Besides diagnosis purposes, the fact that EVs can somehow mirror tumor progression (please see our previous section) raises the possibility that EVs are suitable for theranostic application, personalized medicine and evaluation of response to treatment. In this regard, the levels of exosomal mRNA collected from plasma correlate with the immunological responses of patients enrolled in a vaccination trial. <sup>51</sup> In addition, as mentioned above, MGMT methylation status in circulating EVs was also found to correspond with the identity of the parental tumor and be predictive of the current response to treatment. <sup>52</sup>

Altogether, this recent series of studies hold promise that EVs content may provide a novel tool for early diagnosis and companion biomarkers in combination with the current methods. <sup>10,48</sup>

### **Could EVs be functionally targeted?**

Blocking EV emission, diffusion and transmission may be envisioned as a novel therapeutic strategy. First, an early study demonstrated that the secretory mechanism involved in tumor-based EV release could be halted. <sup>53</sup> Indeed, this was achieved by interfering with the Rab27 small GTPase. Although blocking Rab27 in GBM efficiently reduced migration and invasiveness, this involves slightly different mechanisms, such as lysosomal action. <sup>54</sup> Thus, it is still unclear whether Rab27 contribution to GBM disease relies on EV secretion, as shown in other cancers. <sup>55</sup> On the other hand, exosome uptake can be impaired by heparin. <sup>56</sup> Heparin and  $\alpha$ -difluoromethylornithine (DFMO) target heparan sulfate proteoglycans, impacting the general mechanisms of vesicle endocytosis, including in GBM cells. <sup>57</sup> Cholesterol of lipid rafts on EVs can also be manipulated by methyl- $\beta$ -cyclodextrin (M $\beta$ CD) in the U87 GBM cell line, in order to block lipid raft-mediated endocytosis. <sup>58</sup> Applied to the tumor microenvironment, these approaches could hypothetically affect tumor growth and invasion. However, further efforts must elucidate the signaling mechanisms involved prior to clinical application.

### **Could EVs be used as drug delivery agents?**

Because EVs are prone to protect their content in order to cross membrane/biological barriers, to travel throughout the body and reach specific cellular targets, they emerge as attractive strategy to deliver drugs into the tumor mass, with the idea to target defined cellular components within the tumor microenvironment (*Figure 2*). Paralleling drug delivery systems such as synthetic vectors, adeno-associated virus and lipidic and carbone-based nanocarriers, EVs could be engineered to transport and release therapeutic compounds into the brain.<sup>59</sup> The proof-of-concept has been elegantly established in healthy and Alzheimer-like mouse brains, where systemically injected exosomes were shown to convey siRNA to brain tissues and to specifically and efficiently target RNA expression in neurons, microglia and oligodendrocytes.<sup>60</sup> Dendritic cells were manipulated to release exosomes expressing at their surface Lamp2 fused to the neuron peptide RVG, in order to specifically target neural cells. Meanwhile, siRNA were prior introduced in donor cells.<sup>60</sup> Likewise, exosomes were manipulated to express EGFR ligands and therefore designed to deliver siRNA to EGFR-expressing breast cancer cells.<sup>61</sup> Similarly, intranasal administration of exosomes was proven to efficiently reach brain tissues in mice.<sup>62</sup> In this search for novel therapeutic approaches, systemic injection of brain endothelial cell-derived EVs loaded with cytotoxic drugs (doxorubicin and paclitaxel) were demonstrated to cross the blood brain barrier in a fish model for GBM.<sup>63</sup> In addition, the expression of GBM-specific multidrug transporter could be reduced by anti-miR strategy introduced into exosome-producing donor cells.<sup>64</sup>

Thus, cellular exosomes can be manipulated to specifically reach brain tissues, normally protected against xenobiotic invasion by the blood brain barrier (*Figure 2*). Their content can be specifically enriched with siRNA, miRNA to be delivered to the tumor and even loaded with cytotoxic drugs *via* direct electroporation onto exosome preparation.<sup>56</sup>

Although promising, such data were obtained from *in vitro* approaches and *in vivo* mouse models. To our knowledge, there is no translational application yet in GBM. Preclinical studies should define whether risk for patients can be reduced and therapeutics well tolerated when using non-viable biological structures. To this end, it will be essential to identify safety donor cells (i.e. autologous to the patient, cultured in safety and sterile conditions without derivations...) that can be engineered and loaded with specific cargoes to cross the blood brain barrier and reach the recipient cells.

## **Conclusions and perspectives**

Over the past five years, researchers have collected information on glioblastoma-derived extracellular vesicles in terms of their content and their fundamental properties. Although their genesis and the triage process of cellular component specifically addressed into EVs are rather elusive, it is now well established that circulating EVs reflect tumor state and therefore can serve as diagnostic and theranostic tools towards personalized medicine. Additionally, EVs offer useful features to improve drug delivery systems as they can pass through biological barriers, are immunotolerated, can freely circulate, and their content is protected from degradation. Better knowledge is still however required to clarify their biological functions, their mode of action both within and outside the tumor microenvironment, and their privileged cellular targets.

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No potential conflicts of interest were disclosed.

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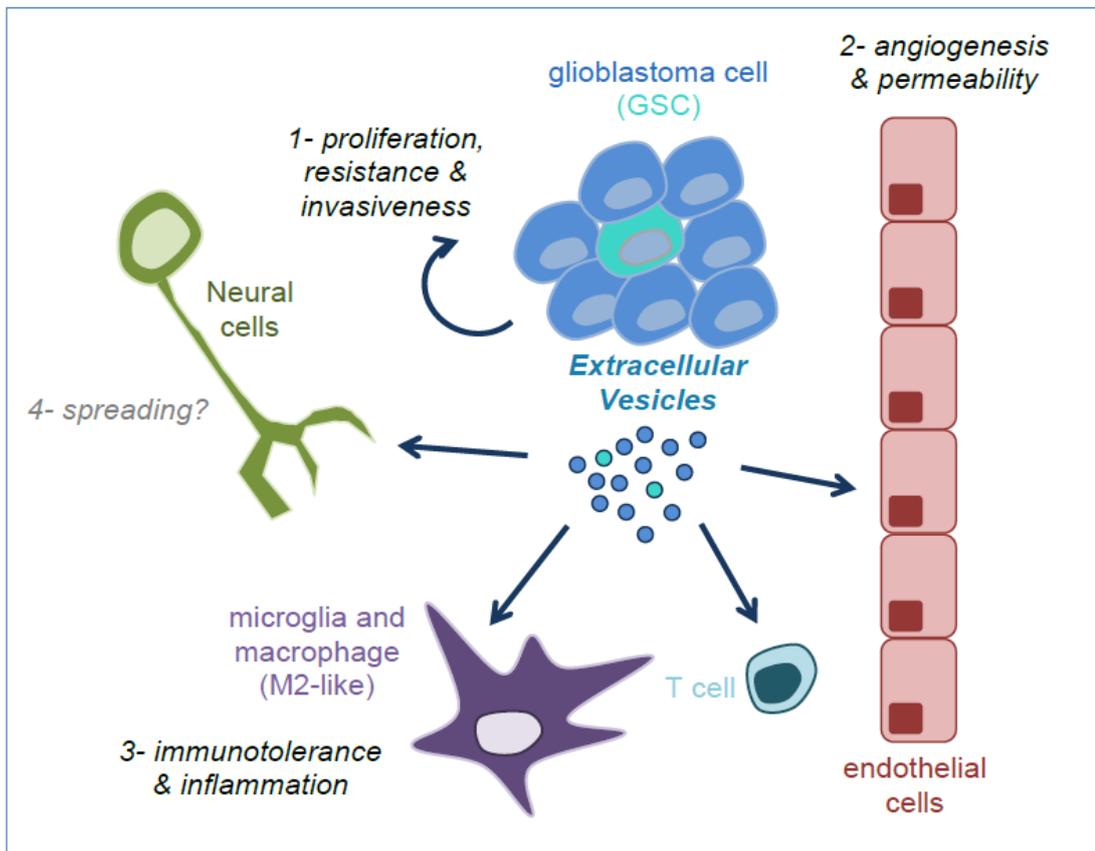
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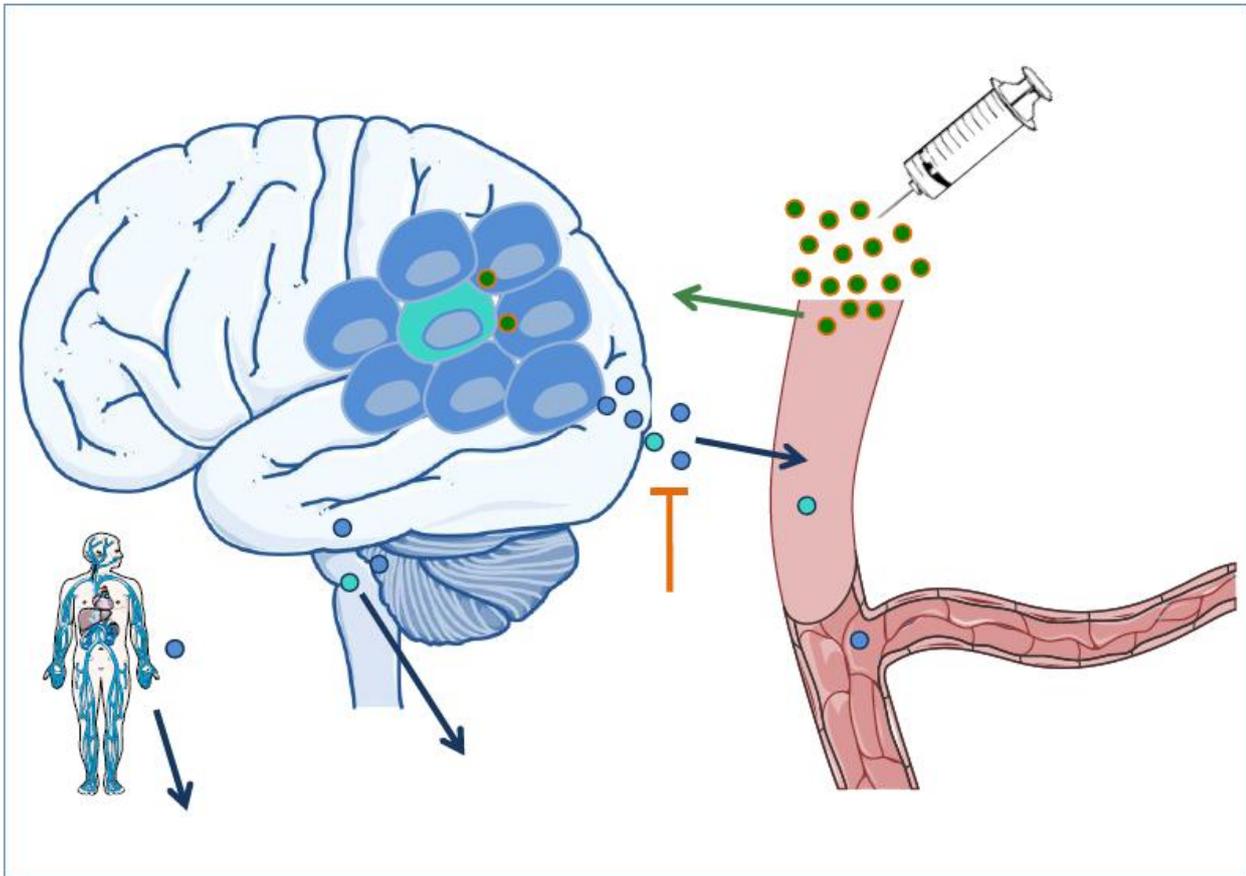
**Table 1. GBM-derived EV cargoes and their functional impacts.**

	Recipient cells	Cargo	Biological effects
<i>Intra-Tumoral</i>	Tumor cells	mtDNA	nd <sup>18</sup>
		miR-1	Tumorigenicity, invasion and growth <sup>34</sup>
		miR-21	Anti-apoptosis, proliferation <sup>10,17</sup>
		hypoxia signature	Cell migration <sup>14</sup>
		EGFRvIII	Tumor growth <sup>12</sup>
		clic1	Tumor growth <sup>65</sup>
		proteins	GBM subtype tumorigenesis <sup>25</sup>
		TrkB	Aggressiveness <sup>50</sup>
	Endothelial cells	miR-9	Migration, angiogenesis <sup>66</sup>
		mRNAs	Angiogenesis <sup>10</sup>
hypoxia signature		Angiogenesis <sup>14</sup>	
angiogenic factors		Angiogenesis <sup>10</sup>	
semaphorin 3A		Permeability <sup>21</sup>	
Microglia	miR-451, miR-21	Cytokine profile <sup>32</sup>	
Monocyte/macrophage			
<i>Extra-Tumoral</i>	Endothelial cells		Pro-coagulation <sup>39,40</sup>
	Endothelial cells	semaphorin 3A	nd <sup>21</sup>
		TrkB	nd <sup>50</sup>
	Monocyte/macrophage	proteins	M2 phenotype <sup>37</sup>
	Lymphocyte	proteins	Immunotolerance <sup>67</sup>

nd: not determined



**Figure 1. Glioblastoma-derived extracellular vesicles actions in the tumor microenvironment.** Glioblastoma and Glioblastoma Stem-like (GSC) cells composing the tumor mass release extracellular vesicles that can be taken up and affect tumors cells themselves or neighboring cells such as endothelial cells and immune system cells to corrupt their functions (labelled as #1-3). They could also potentially affect healthy surrounding neural cells, such as neurons and astrocytes, and therefore spread oncogenic materials (indicated as #4).



**Figure 2. Potential translational applications of extracellular vesicles in glioblastoma.** Extracellular Vesicles (EVs) are not yet used in clinic for glioblastoma patients but recent researches highlight them as promising circulating biomarkers, potentiate new tools for drug delivery and new target to block intercellular communication.